SYNTHESIS, DNA INTERACTIONS AND ACTIVATION OF NOVEL CYTOTOXIC ANTHRAQUINONES

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DECLARATION

The work reported in this thesis is original, except where due reference is made, and has not been submitted in whole or in part to any other degree awarding body or institution.

CONTENTS

TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 4

CHAPTER 5

FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

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ABSTRACT

Mitoxantrone is a dihydroxyanthracenedione derivative with significant clinical activity against advanced breast cancer, lymphoma and several types of leukaemia. However, as with all conventional anticancer agents, its non-specific nature results in dose-limiting systemic toxicity. The pharmacological (and toxicological) effects of mitoxantrone are thought to be mediated through its interaction with DNA and the DNA processing enzyme, topoisomerase II. Hence, an investigation of the DNA binding properties of mitoxantrone and related bis-substituted alkylaminoanthraquinones was undertaken with a view to developing agents with reduced toxicity to normal cells. DNA intercalation was evaluated using spectrophotometric and DNA thermal denaturation (Tm) techniques with calf thymus DNA. Overall, compounds that were hydroxylated at the 5 and 8 positions of the anthraquinone chromophore had increased DNA binding affinity compared to their non-hydroxylated analogues. The ΔTms of mitoxantrone and its non-hydroxylated analogue ametantrone, were 26.4°C and 21.5°C respectively. The affinity constants (K) for the chromophore-hydroxylated compounds were between 3.94 and 4.95 $x10^6M^{-1}$ while K values for their non-hydroxylated analogues were between 1.63 and 3.25 $x10^6M^{-1}$. For the di-N-oxide, AQ4N, intercalation was not detectable. The mono-N-oxide, AQ6N, showed modest DNA binding activity, with a ΔTm of 7.0°C and an affinity constant of 3.64 $x10^6M^{-1}$. Several of the alkylaminoanthraquinones were further investigated for their ability to inhibit decatenation of kDNA by topoisomerase II. Mitoxantrone and its analogues, AQ4 and AQ6, inhibited decatenation at concentrations of 0.75μM, 1.5μM and 1.0μM respectively. In contrast, total inhibition of decatenation by the N-oxides, AQ4N and AQ6N, required concentrations of 50μM and 10μM respectively. Hence modification of the terminal nitrogen on both alkylamino side chains to form a di-N-oxide resulted in a large decrease in DNA binding affinity and topoisomerase II inhibition. In view of the importance of the cationic alkylamino side chains in intercalative binding and the enhanced ability of mitoxantrone to inhibit topoisomerase II, a series of acetalanthraquinones were synthesized. These compounds, referred to as YCG7 (1-substituted), YCG8 (1,4-bis-substituted), and YCG9 (1,5-bis-substituted), possessed dimethoxy groups in place of the alcohol groups of mitoxantrone, and were designed to be converted to their respective aldehydes, which should increase their cytotoxic activity due to their potential to form Schiff's bases with intracellular targets. The acetalanthraquinones were relatively poor DNA intercalators compared to the parent compound, ametantrone. The ΔTm values for YCG7, YCG8 and YCG9 were 3.6 °C, 14.2 °C and 15.7 °C respectively. The bathochromic shifts for the acetalanthraquinones in the presence of calf thymus DNA and 0.5M NaCl/0.008M Tris at a DNA:drug ratio of 10:1 were 1.4nm, 3.5nm and 3.6nm respectively for YCG7, YCG8 and YCG9 (ametantrone=12.2nm). Oxidative metabolism by NADPH-fortified mouse liver microsomes provided an effective route for the oxidation of acetalanthraquinones, producing two polar metabolites for YCG7 and three polar metabolites for both YCG8 and YCG9. The metabolites of YCG7 and YCG8 were considerably more cytotoxic against the V79 Chinese hamster lung cell line than their respective acetalanthraquinones (4.4-fold for both compounds). YCG9 was found to be relatively cytotoxic *per se*, possibly due to its different mode of DNA binding, involving 'straddling' of the DNA helix. The increase in cytotoxicity of the metabolized products compared with the acetalanthraquinones supports the concept that they were converted to their corresponding aldehydes. This leads the way to the design of acetal-containing cytotoxic agents which can be selectively activated in tumours.

CHAPTER 1: INTRODUCTION

1.1 CANCER

Cancer can be defined as a collection of diseases characterized by uncontrolled cell proliferation leading to dissemination (metastasis) to other parts of the body, which eventually compromises normal body function. The spread of primary growths to secondary locations distinguishes cancer from benign tumours and makes its eradication far more difficult. In the European Community, the current mortality rate from cancer is 1 in 4 deaths, a figure set to rise. The commonest malignancy in British women is breast cancer, which affects 1 in 12, whereas in the Greek population the number is only half that (Cancer Research Campaign Scientific Yearbook, 1993). Contrary to the belief that various populations are genetically predisposed to particular types of cancer, incidence rates are largely dependent on environmental factors such as diet and lifestyle.

Carcinogenesis, the process leading to cancer formation, is complex and not yet fully understood, but often involves phenotypic alterations that give cancer cells a selective advantage for growth over normal cells. These may include overcompetitive behaviour for growth factors, overproduction of intracellular growth signals or errors in genes which regulate programmed cell death (apoptosis) e.g. bcl-2, c-myc, p53 (see reviews by Ellis *et al*, 1996; Dixon *et al*, 1997). This is usually accompanied by modifications which enable cells to invade surrounding tissue and metastasize to other parts of the body to form new tumours (King, 1996). Due to a high level of genetic instability, generation of errors continues as the cancer progresses.

The prognosis for patients treated when their cancer is in an early stage is invariably better, due to the smaller number and less malignant nature of the tumour. Surgery is the usual starting point for treatment of solid tumours, followed by either radiation therapy and/or chemotherapy, depending on the site and nature of the disease. As a majority of tumours cells have a higher proportion of dividing cells than normal tissue, they are generally more susceptible to the lethal effects of chemotherapeutic agents. However, rapidly dividing normal cells, such as those of the bone marrow,

digestive tract and hair follicles are also affected, causing unwanted side effects such as myelosuppression, vomiting, gastrointestinal disturbances and alopecia (see section 1.4.1). This poses limitations on the dose of a particular drug that can be administered, which in turn reduces the effectiveness of the treatment.

The problem of systemic toxicity has been partially alleviated by combination therapy, which usually involves the administration of two or three cytotoxic agents with different mechanisms of action. This strategy also enhances overall tumour cell kill, but rarely eradicates all cells, and the small clones of progenitor cells which survive treatment frequently redivide to form a new, drug resistant tumour (section 1.4.2). Unfortunately, this means that with the exception of prostate cancer, Hodgkin's lymphoma and childhood leukaemia, cancer is often fatal. As a consequence, extensive research is being carried out to find new drugs that overcome the problems of toxicity and resistance associated with chemotherapy (see section 1.5). Many of these are based on compounds already in clinical use.

1.2 DNA INTERCALATING AGENTS

Among the agents currently used in the treatment of cancer are the DNA intercalating agents. These possess a polycyclic aromatic ring system that allows reversible non-covalent interdigitation between adjacent base pairs in the hydrophobic interior of the DNA double helix (Lerman, 1961). This results in local distortion and an overall lengthening of the DNA helix as it unwinds to accommodate the drug molecule (Waring, 1970; Reinert, 1983; figure 1.1). Compounds with tri- and tetracyclic chromophores, which are of approximately the same dimensions as the purine-pyrimidine base pairs, show optimal binding. In some cases, the drug may contain additional constituents attached to the chromophore which strengthen intercalation by interacting with the anionically charged DNA phosphate backbone (Foye *et al*, 1982; Pachter *et al*, 1982; Pohle *et al*, 1990). However, this type of binding is much weaker than intercalation because it occurs on the exterior surface of the DNA helix, and is thus

influenced to a large extent by the conditions of the surrounding medium (Jones *et al*, 1980).

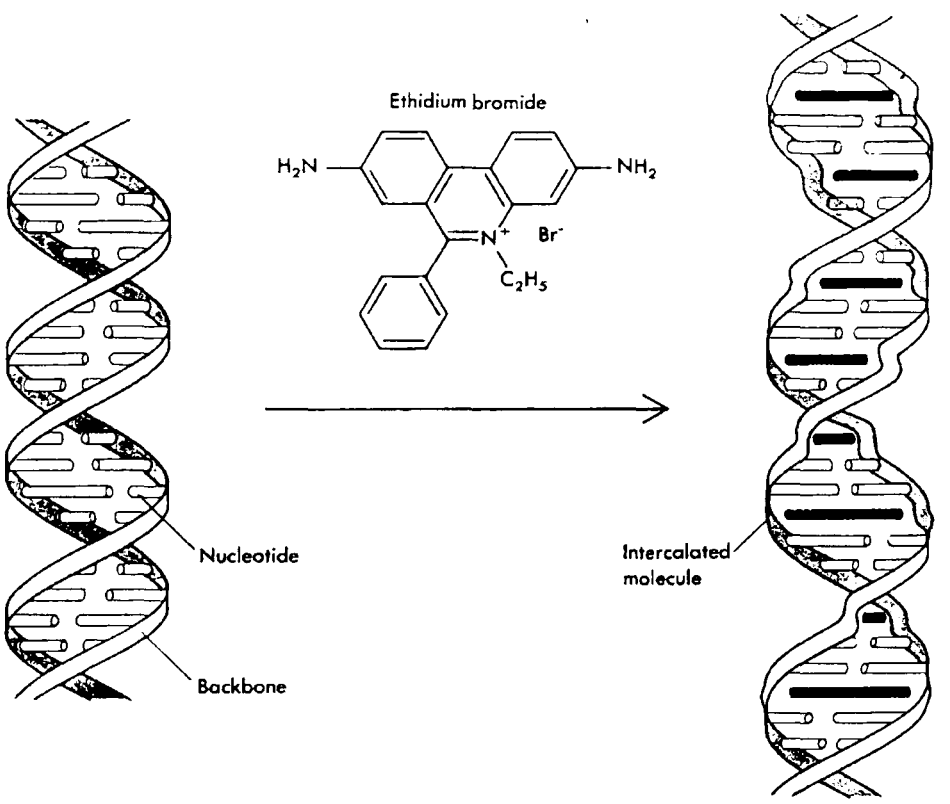
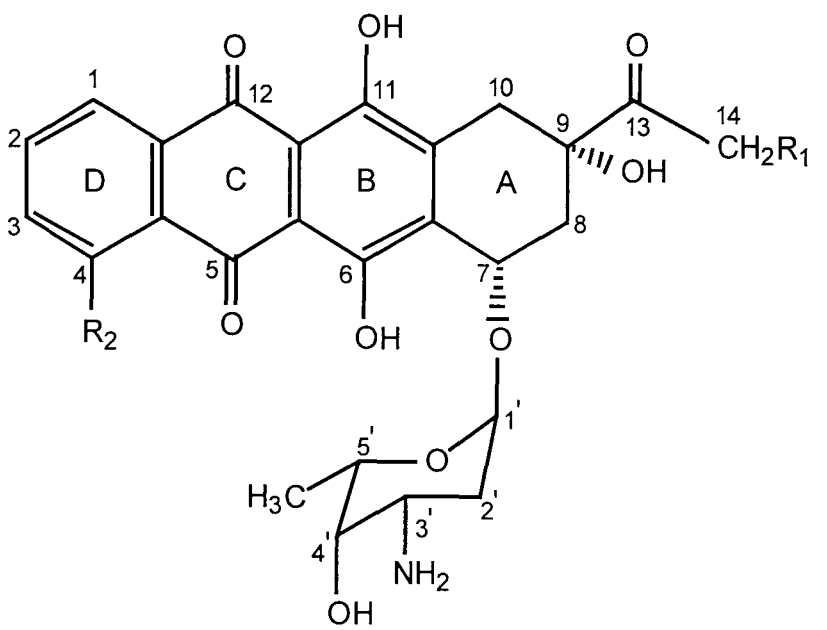


Figure 1.1: Intercalation of ethidium bromide into double-stranded DNA, showing lengthening of the DNA helix (reproduced from Watson *et al*, 1987).

1.2.1 THE ANTHRACYCLINE ANTITUMOUR AGENTS

The anthracycline antibiotics daunorubicin and doxorubicin were isolated from *Streptomyces* species in the 1960s, and are still among the most widely used and effective chemotherapeutic agents in the clinic today (for a review, see Lown, 1993). Chemically, the anthracyclines contain an amino sugar attached to an aglycone ring, the latter consisting of a non-aromatic (A) ring conjugated with an anthraquinone moiety (figure 1.2). Doxorubicin is the 14-hydroxy derivative of daunorubicin, and this structural modification significantly increases its spectrum of activity. Thus, while the use of daunorubicin is limited to the treatment of haematological malignancies (Bassan

et al, 1996), doxorubicin can also be used alone or in combination to treat a wide range of solid tumours, including cancers of the breast (Fisher *et al*, 1989) and ovary (A'Hern & Gore, 1995).



Doxorubicin: R_1= OH; R_2= OCH_3

Daunorubicin: R_1= H; R_2= OCH_3

Idarubicin: R_1= R_2= H

Epirubicin: R_1= OH; R_2= OCH_3; OH at 4' = equatorial

Figure 1.2: The antitumour anthracyclines doxorubicin and daunorubicin, and their analogues, epirubicin and idarubicin.

The principal mode of action of the anthracyclines is thought to be mediated through intercalation of the aglycone portion of the molecule between adjacent DNA base pairs. Intercalation occurs preferentially between CA/T bases with the chromophore perpendicular to the base pair axes and the daunosamine sugar positioned

in the minor groove (Frederick *et al*, 1990; Gao & Wang, 1991). This presumably provides a means for the inhibition of DNA- and RNA polymerase activities observed for the anthracyclines, resulting in the cessation of DNA and RNA synthesis respectively (Meriweather & Bachur, 1972; Zunino *et al*, 1975). It also allows doxorubicin and daunorubicin to stabilize the covalent complex formed between DNA and topoisomerase II during the strand passage reaction (see section 1.3.2.1).

In addition to its intercalative mechanism of action, doxorubicin can mediate cytotoxicity without entering L1210 leukaemia cells (Tritton & Yee, 1982). This has raised the possibility that it can mediate cell death purely through activating signal transduction pathways at the plasma membrane (Tritton, 1991). Doxorubicin and daunorubicin also produce DNA damage through the generation of free radicals, although the importance of this process in their antitumour activity remains controversial and is generally considered to be more closely associated with their undesirable side effects, particularly their severe cardiotoxicity, which can be dose-limiting (see section 1.4.1.2).

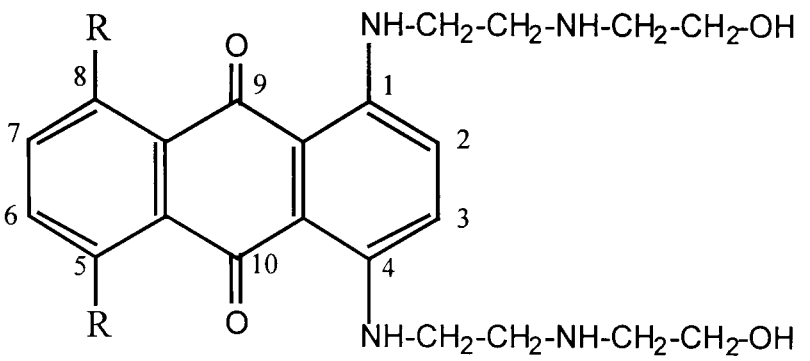
As a result of the highly undesirable features associated with the use of the anthracyclines in chemotherapy, much time and effort has been devoted to developing derivatives of doxorubicin and daunorubicin in the hope of enhancing antitumour activity and/or lowering toxic side effects. Some of the most successful derivatives include epirubicin (4'-epidoxorubicin) and idarubicin (4-demethoxydaunorubicin) which are currently available for the treatment of advanced cancers (Cersosimo, 1992; Bonadonna *et al*, 1993; see figure 1.2). Unfortunately, despite the reduced cardiotoxicity of epirubicin (see section 1.4.1.2) and the superior activity of idarubicin compared to doxorubicin and daunorubicin in several cell lines (Supino *et al*, 1977; Curtis *et al*, 1995), neither of these analogues has an improved therapeutic index or a significantly different spectrum of activity to doxorubicin *in vivo*.

In the 1970s, it was surmised that the unwanted cardiotoxic effects of the anthracyclines may be caused by their amino sugar. This supposition was based on the

observation that the aglycones of digitalis produced more transient and less potent myocardial damage than the glycosides (Adamson, 1974). However, it was also noted that if separated, neither the aglycone nor daunosamine sugar of the anthracyclines retained antitumour activity (Zee-Cheng & Cheng, 1970). Wishing to design new series of compounds, two research groups (Zee-Cheng & Cheng, 1978; Murdock *et al*, 1979) decided to remove the daunosamine sugar and replace the amino function, which was essential for activity, with another, appropriately positioned amino group. In addition, the non-aromatic portion of the molecule was also omitted, since this was not required for intercalation (or activity) and caused synthetic complications due to its stereochemical configuration. An intensive screening programme of compounds incorporating these structural modifications soon ensued.

1.2.2 THE ANTHRAQUINONE ANTITUMOUR AGENTS

The two most successful anticancer agents to emerge from the 1970s screening programme were the bis-substituted alkylaminoanthraquinones mitoxantrone and ametantrone (figure 1.3). Both these compounds contain 2'-(hydroxyethylamino)-ethylamino side chains at the 1 and 4 positions of the chromophore, but differ in the fact that mitoxantrone is hydroxylated at the 5 and 8 positions, whereas ametantrone is unsubstituted. This structural alteration is fundamental to the 10 to 50-fold increase in potency of mitoxantrone compared with ametantrone *in vivo* (Johnson *et al*, 1979; Krapcho *et al*, 1991) and its activity against advanced breast cancer, non-Hodgkin's lymphoma and several types of chronic and acute leukaemia (reviewed by Shenkenberg & von Hoff, 1986, Faulds *et al*, 1991). Mitoxantrone also has superior antitumour efficacy to compounds where the methylene bridge spacer group is more than two carbons in length, compounds with side chains not containing a terminal nitrogen, or those containing a primary or tertiary terminal nitrogen (see Cheng & Zee-Cheng, 1983; Randall *et al*, 1983).



mitoxantrone: R=OH
ametantrone R=H

Figure 1.3: The anthraquinone antitumour agents mitoxantrone and ametantrone.

In common with the anthracyclines, biochemical evidence indicates that nucleic acids are among the principal targets of the anthraquinones and that DNA-drug interactions contribute significantly to cytotoxic activity. Confocal imaging studies on drug uptake and distribution in intact human cells have shown that mitoxantrone accumulates in the nucleus, particularly the nucleolus (Smith *et al*, 1992; Fox & Smith, 1995; Feofanov *et al*, 1997). This is consistent with preferential binding of the drug to nucleolar DNA and condensation of ribopolymers (Chegini & Safa, 1987). A considerable proportion of the drug is also contained within small cytoplasmic inclusions grouped around the periphery of the nucleus, where it is sequestered for long periods. Mitoxantrone is also associated with the hydrophobic cytoskeleton (Cress *et al*, 1988; Roberts *et al*, 1989) and along these lines, it has been reported that it can mediate its intracellular effect through the inhibition of microtubule assembly (Ho *et al*, 1991).

The majority of theoretical and experimental data indicate that 1,4-bis-substituted alkylaminoanthraquinones intercalate with the chromophore perpendicular to the long axes of the base pairs and both side chains projecting into the major groove extending

in the 5' direction (Lown & Hanstock, 1985; Chen *et al*, 1986; Fox *et al*, 1986). In this configuration, the cationic amino groups of the side chains are believed to interact with DNA via the negatively charged DNA phosphate groups which stabilizes the DNA-drug interaction (Lown & Hanstock, 1985). The most recent evidence, obtained by means of a novel transcriptional assay, indicates that intercalation of mitoxantrone occurs preferentially at 5'-(A/T)CA and 5'-(A/T)CG sites (Panousis & Phillips, 1994) with some indication that binding to the latter of these sites is dependent on the two chromophore hydroxyl groups (Bailly *et al*, 1996).

Numerous studies have been conducted in an attempt to shed light on molecular basis for the superior activity of mitoxantrone compared with its analogues, with some interesting results. For example, mitoxantrone-DNA intercalation complexes dissociate three or four times more slowly than those of ametantrone, due to prolonged retention of the mitoxantrone chromophore in the hydrophobic interior of the double helix (Denny & Wakelin, 1990). This has been attributed to the bulky nature of hydroxy groups (Denny & Wakelin, 1990) and the increased lipophilicity of the mitoxantrone chromophore (Patterson, 1993). More recently, Mazerski *et al* (1998) have suggested that the increase in chromophore width conferred by the hydroxy groups may keep the drug in a more coplanar orientation within the intercalation site, resulting in greater electrostatic interactions with the DNA base pairs. The slower dissociation of mitoxantrone from DNA may increase its cytotoxicity by prolonging the arrest of DNA and RNA synthesis (Fox & Smith, 1990) and by enhancing stabilization of the DNA-topoisomerase II cleavable complex (see section 1.3.2).

The pharmacological activity of mitoxantrone and ametantrone may also be mediated through their ability to form inter-DNA cross-links, which leads to compaction and distortion of chromatin (Lown *et al*, 1984; Kapuscinski & Darzynkiewicz, 1986). This property is attributed to the alkylamino side chains containing two basic (amino) groups separated by two carbons, which promotes electrostatic interactions between the drug and DNA (Lown *et al*, 1984). However, the

process of cross-linking is also influenced by the chromophore hydroxy groups, inasmuch as mitoxantrone condenses nucleic acids at 5-40-fold lower concentrations than ametantrone, another factor which may contribute to their difference in potency (Kapuscinski & Darzynkiewicz, 1986).

Early evidence indicated that mitoxantrone was significantly less cardiotoxic than doxorubicin and daunorubicin (see reviews by Shenkenberg & von Hoff, 1986; Faulds *et al*, 1991). This was attributed partly to the fact that it lacks the amino sugar moiety and tetracyclic A ring of the anthracyclines, and partly to its more negative reduction potential in aqueous solution (-0.79V) compared to the anthracyclines (-0.6V). The lower reduction potential of mitoxantrone diminishes its ability to undergo cytochrome P-450-mediated reductive activation to generate free radicals (Sinha *et al*, 1983; Nguyen & Gutierrez, 1990) a process that has been connected with the cardiotoxicity of the anthracyclines (see section 1.4.1.2). However, recent studies have shown that mitoxantrone is considerably more cardiotoxic than was previously suggested (Andersson *et al*, 1999; see section 1.4.1.3).

1.2.3 INVOLVEMENT OF BIOTRANFORMATION IN THE ANTITUMOUR ACTIVITY OF MITOXANTRONE

Many attempts have been made to characterize the biotransformation of mitoxantrone, and it is now well established that the two major metabolites of this drug in humans are the mono- and dicarboxylic acid derivatives, resulting from oxidation of the alcohol groups of the alkylamino side chains (figure 1.4). These derivatives have been detected in urine and plasma samples from cancer patients (reviewed by Ehninger *et al*, 1990) and are both devoid of antitumour activity (Chicarelli *et al*, 1986). In addition, several other metabolites of mitoxantrone have been detected which are not well characterized. Using a high-performance liquid chromatography method, Ehninger *et al* (1986) discovered a metabolite in urine and serum samples of breast cancer patients which was more polar than mitoxantrone, but less polar than either of the

carboxylic acid derivatives. Up to several hours after drug administration, this compound was the predominant metabolite. In contrast, the levels of the carboxylic acid derivatives did not peak until some time later and were still present in urine samples taken 4 weeks after treatment. The fact that peak levels of the carboxylic acid derivatives were not reached for a considerable time after drug administration suggests a sequence of metabolic events could be involved in their formation. Conceivably, the third metabolite may be a precursor of the carboxylic acid derivatives, since it is less polar than either of these derivatives but more polar than mitoxantrone, and is comparatively short-lived.

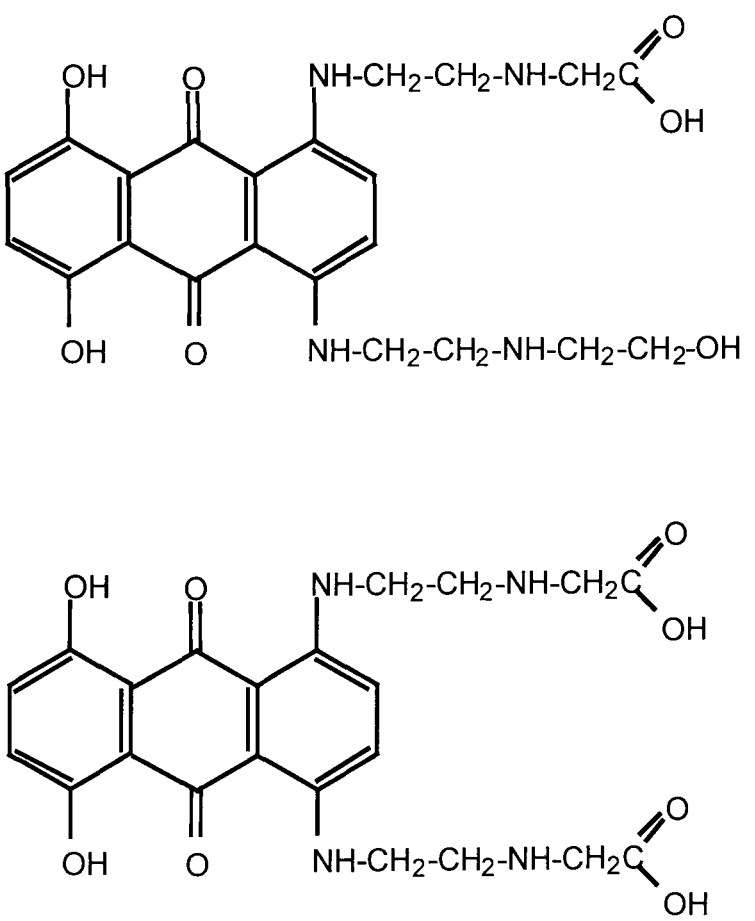


Figure 1.4: The mono- and di-carboxylic acid metabolites of mitoxantrone, produced by oxidation of the alcohol groups of the side chains.

Using human hepatocytes in primary culture, Richard *et al* (1991) obtained a similar pattern of metabolism of mitoxantrone to the one described by Ehninger *et al* (1986). However, in rat hepatocytes the predominant metabolites were found to be two highly polar compounds, corresponding to the glutathione- and glucuronide-conjugates of mitoxantrone whereas only trace amounts of the carboxylic acid derivatives were present. These conjugates were also detected in the human samples, but represented only minor amounts of drug product. The biotransformation pathway in rabbits was also different from that in humans, indicating a large interspecies variability exists in the metabolism of mitoxantrone. Even within humans, the levels of mono- and dicarboxylic acid metabolites excreted varies considerably (Ehninger *et al*, 1986; Schleyer *et al*, 1994). This has led to the speculation that interindividual variation in the formation of metabolites might underlie differences in clinical response to mitoxantrone.

Only one other mitoxantrone metabolite has been detected in significant amounts in human urine, by Blanz *et al* (1991a). This has been identified as the tetrahydronaphthoquinoxaline (TNQ) derivative, formed by intramolecular cyclization of one of the alkylamino side chains in the oxidized, diiminoquinone form of the drug (figure 1.5). The TNQ derivative can also be generated *in vitro* by horseradish peroxidase-catalysed oxidation of mitoxantrone with hydrogen peroxide (Reszka *et al*, 1986; Kolodziejczyk *et al*, 1988). This occurs via the formation of free radical intermediates, and produces DNA damage (Fisher & Patterson, 1991) and DNA cross-links (Reszka *et al*, 1989). Several other enzymes have been shown to generate the TNQ derivative, including a human lactoperoxidase/hydrogen peroxide system (Reszka *et al*, 1997), and a myeloperoxidase-dependent mechanism in human neutrophils (Panousis *et al*, 1994). In the latter system, TNQ formation was accompanied by covalent incorporation of the drug into cellular DNA and RNA (Panousis *et al*, 1995, 1997).

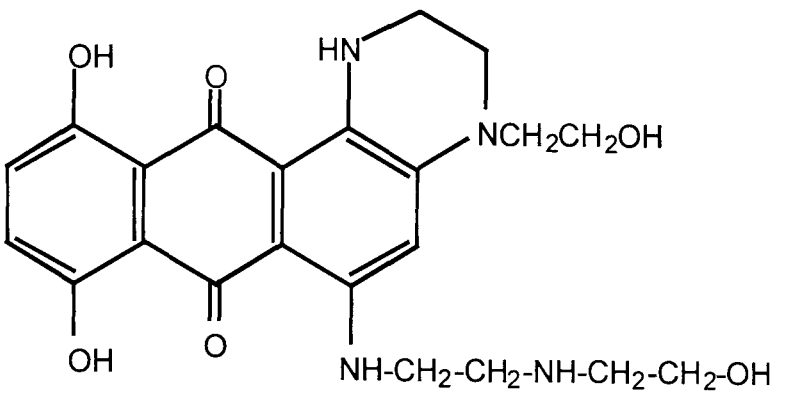


Figure 1.5: The TNQ metabolite of mitoxantrone.

Formation of the TNQ derivative in HepG2 cells is inhibited by metyrapone, demonstrating that oxidative activation is cytochrome P-450-dependent (Mewes *et al*, 1993; Zeller *et al*, 1994). The cytotoxicity of mitoxantrone in HepG2 cells is also significantly reduced in presence of metyrapone (Duthie & Grant, 1989; Zeller *et al*, 1994) which indicates, at least *in vitro*, that oxidative activation of mitoxantrone is involved in its mechanism of action. However, as the presence of metyrapone is also likely to inhibit other possible routes of cytochrome P-450- dependent oxidative metabolism of mitoxantrone, it can not be unequivocally proven that the TNQ is the only active metabolite, and thus its contribution to the cytotoxicity of mitoxantrone *in vivo* remains unclear. In any case, most evidence indicates the principal mechanism of action of the alkylaminoanthraquinones is related to DNA intercalation and subsequent stabilization of the DNA-topoisomerase II cleavable complex.

1.3 DNA TOPOISOMERASES AS TARGETS FOR CHEMOTHERAPY

1.3.1 THE STRUCTURE AND FUNCTION OF DNA TOPOISOMERASES

In mammalian cells, genetic material exists as an intricate series of coils and loops arranged so that the ends of the DNA strands are tightly anchored to the nuclear matrix (Verheijen *et al*, 1988). While maintaining this high level of organization, the DNA strands must be able to unwind in order for events such as transcription and replication to occur, and must also be able to separate at cell division. This is achieved by topoisomerases, enzymes which catalyse topological and conformational changes to DNA through the introduction of transient DNA strand breaks.

Human cells contain four types of topoisomerase: topoisomerase I, a 100KDa monomeric protein encoded by a single copy gene located on chromosome 20q12-13.2 (Juan *et al*, 1988), topoisomerase III, encoded by a single copy gene located on chromosome 17p11.2-12 (Hanai *et al*, 1996) and two isoforms of topoisomerase II (α and β). Topoisomerase I and topoisomerase III both achieve conformational changes to DNA by passing a single-stranded DNA segment through a transient single-stranded break made in the complementary strand (Wang, 1996; Li & Wang, 1998). In contrast, topoisomerase II enzymes each consist of homologous dimers and are characterized by their ability to transport a DNA duplex through the double-stranded break of a second DNA duplex, an event known as strand passing (reviewed by Watt & Hickson, 1994, Wang, 1996). As a result, they can resolve catenanes and knots in DNA as well as relaxing supercoils, and since decatenation is essential for the separation of replicated DNA at mitosis and meiosis, topoisomerase II is essential for the viability of all eukaryotic cells (Holm *et al*, 1989; Downes *et al*, 1991).

Until the discovery of the 180kDa topoisomerase IIβ by Drake *et al* (1987), it was thought that only the 170kDa isoform of topoisomerase II existed in eukaryotic cells (now known as topoisomerase IIα) and at present this is still believed to be the case for yeast (Hammonds *et al*, 1998). The single-copy gene for human topoisomerase

IIβ has since been mapped to chromosome position 3p 24 (Jenkins *et al*, 1992), and its topoisomerase IIα counterpart to 17q 21.22 (Tsai-Pflugfelder *et al*, 1988). The two isoforms possess distinct biochemical properties, nuclear distribution, cell cycle regulation and biological roles (Drake *et al*, 1989; Woessner *et al*, 1991; Danks *et al*. 1994). Nonetheless, structurally speaking they are quite similar, and the topoisomerase II monomer can be divided into three distinct domains (Caron & Wang, 1993). The N-terminal domain contains the consensus sequences for ATP binding, while the central domain contains the tyrosine residue that forms the covalent linkage with DNA during the strand passage reaction, located at amino acid position 804 for topoisomerase IIα and 821 for topoisomerase IIβ (Beck *et al*, 1993; Withoff *et al*, 1996). The main difference between the two isoforms resides in the C-terminal domain, which is longer for topoisomerase IIβ than for topoisomerase IIα (Watt & Hickson, 1994). This region contains the serine and threonine residues phosphorylated by enzymes such as casein kinase II and protein kinase C, which regulates topoisomerase II catalytic activity (Enomoto *et al*, 1993). There is also mounting evidence that the C-terminal plays a role in enzyme dimerization and nuclear localization (Watt & Hickson, 1994).

Based on crystal structure information and several pieces of biochemical evidence (Roca & Wang, 1992; Roca *et al*, 1996), a two gate model has been proposed for the strand passage reaction of topoisomerase II (Berger *et al*, 1996). The two gates are provided by dimerization of the topoisomerase II monomers to form a heart shaped structure with a large, hydrophobic hole at the centre (figure 1.6). The resulting ATP-modulated clamp permits tightly controlled passage of an intact DNA duplex (T-segment) through a cleaved DNA duplex (G-segment). The topological integrity of the cleaved DNA segment is maintained during strand passing by transesterification between a pair of tyrosyl residues, one in each half of the topoisomerase II dimer, and a pair of DNA phosphoryl groups, staggered by four base pairs on opposite DNA strands. The phenolic oxygens of the tyrosine residues are covalently linked to the phosphoryl groups at the 5' end of the strands, leaving a pair of hydroxyl groups on the recessed 3'

ends. The covalent DNA-protein complex is described as the cleavable complex, as denaturation of this complex with NaOH or SDS leads to protein-linked DNA breaks (see section 1.3.2.1). Although strand passage can be achieved using non-hydrolysable ATP analogues, enzyme turnover absolutely requires hydrolysis of ATP (Osheroff, 1986). Enzyme activity also requires the presence of a divalent metal cation, usually Mg^{2+} (Osheroff, 1987).

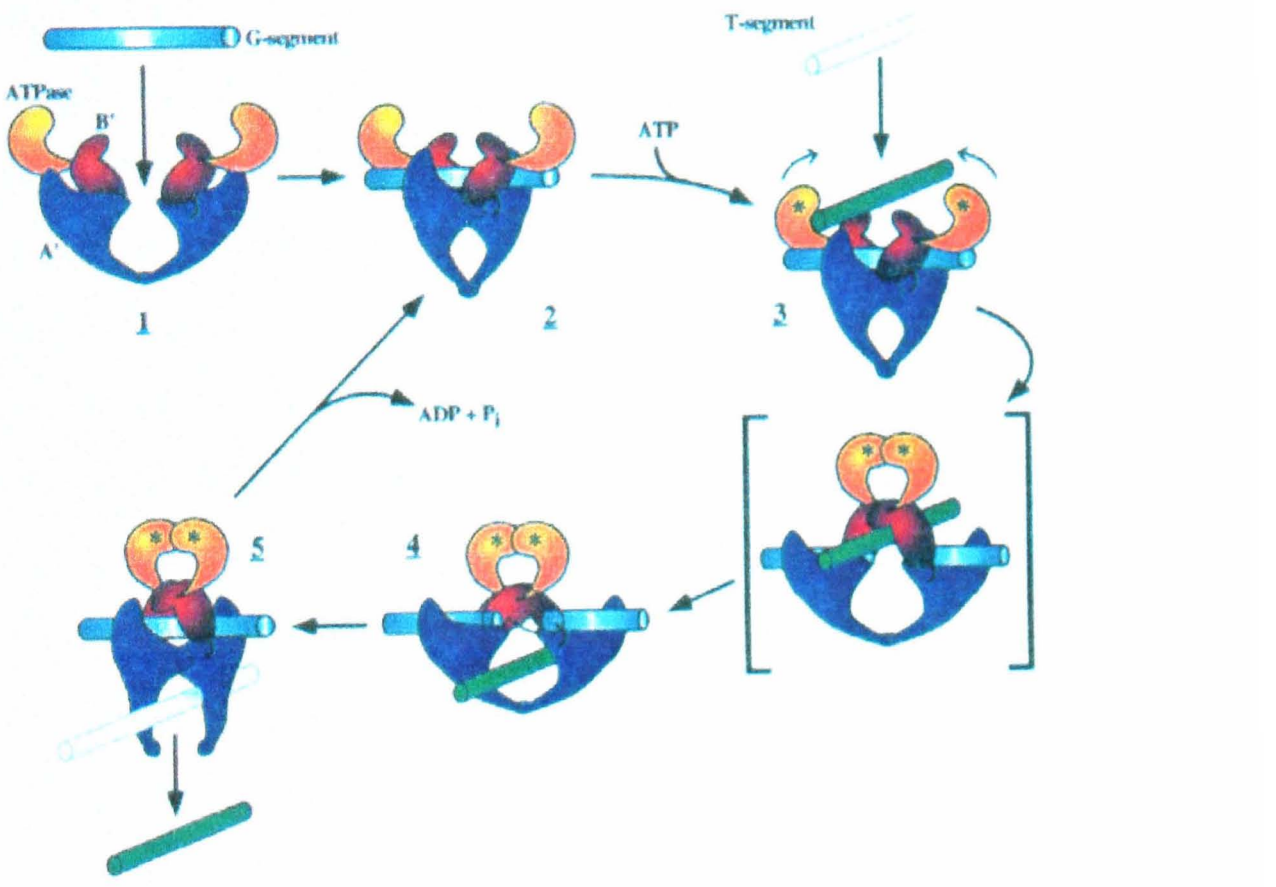


Figure 1.6: A two-gate mechanism for the catalytic reaction of topoisomerase II. Reproduced from Berger *et al*, 1996.

1.3.2 INHIBITION OF TOPISOMERASES

1.3.2.1 TOPOISOMERASE POISONS

One of the major advances in cancer research over the past two decades has been the discovery that topoisomerases are inhibited by a wide variety of chemical agents, including a number of those currently employed in chemotherapy (reviewed by Wang *et al*, 1997, Gatto *et al*, 1999). The most fully characterized topoisomerase I inhibitors are the plant alkaloid camptothecin, originally isolated from the Chinese tree *Camptotheca acuminata* (Wall *et al*, 1966) and several of its analogues, which show promise against human solid tumours previously refractive to chemotherapy (see Muggia *et al*, 1996).

The main types of topoisomerase II inhibitors can be catagorised according to their modes of action against the enzyme (figure 1.7). Topoisomerase II-binding inhibitors act on the enzyme directly, by preventing protein conformational transitions. In contrast, DNA-binding inhibitors hinder binding of the enzyme to the DNA substrate. However, DNA intercalators such as doxorubicin, mitoxantrone, and non-intercalating agents such as the epipodophyllotoxins, exert their effect by stabilizing the covalent DNA-topoisomerase II cleavable complex formed during the strand passage reaction (Chen *et al*, 1984; Nelson *et al*, 1984; Tewey *et al*, 1984a, 1984b). Due to this mechanism of action, they are known as topoisomerase poisons, as their cytotoxicity is thought to stem from interference with essential metabolic processes such as replication and transcription, which literally 'poisons' the cell (see section 1.3.2.3). The addition of a protein denaturant such as SDS or alkali to the drug-trapped complex generates protein-associated single- and double stranded breaks consisting of topoisomerase II linked covalently to the DNA via a phosphotyrosine linkage at the 5' termini (Zhang *et al*, 1990; figure 1.8).

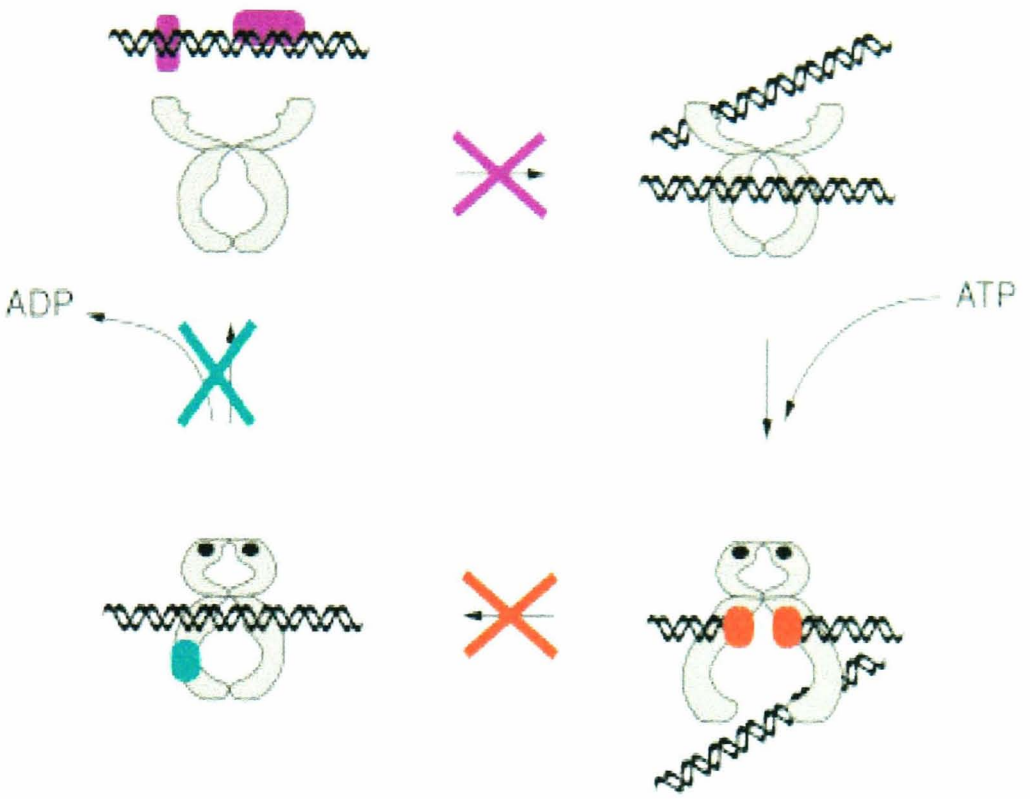


Figure 1.7: The catalytic cycle of type II DNA topoisomerases, showing the modes of action of the inhibitors and poisons of the enzyme. DNA-binding inhibitors, such as groove binders, hinder binding of the enzyme to the DNA substrate (purple); topoisomerase II-binding inhibitors such as merbarone, aclarubicin and fostriecin act on the enzyme directly, by trapping the closed clamp conformation of the enzyme in the presence of ATPase (green); topoisomerase II poisons exert their effect by stabilizing the covalent DNA-topoisomerase II cleavable complex formed during the strand passage reaction (red). Reproduced from Capranico *et al*, 1997.

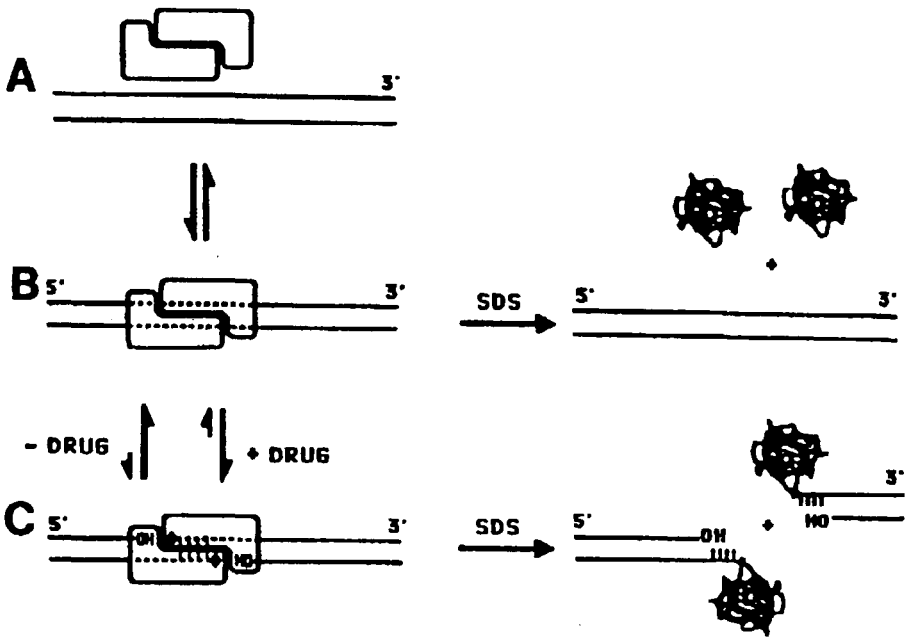


Figure 1.8: Formation of protein-associated double strand breaks following denaturation of the DNA-topoisomerase II cleavable complex: During the topoisomerase II catalytic reaction, the enzyme and DNA (A) combine to form the noncleavable complex (B, left) and subsequently the cleavable complex (C, left) which are all at equilibrium. Exposure of the cleavable complex to SDS results in the generation of DNA double strand breaks with their 5'phosphoryl termini covalently linked to the denatured enzyme (C, right). In contrast, exposure of the noncleavable complex to SDS results in the regeneration of intact DNA strands, which are not associated with the enzyme (B, right). Adapted from Zhang *et al*, 1990.

1.3.2.2 STRUCTURAL REQUIREMENTS FOR DRUG ACTIVITY

Investigations regarding the molecular aspects of anthracycline interference with topoisomerase II have established that the substituents at the C-7 and C-9 positions are the two main participants. At C-7, activity against the enzyme is determined by the nature of the substitutent at the 3'-N position of the daunosamine sugar (Bodley *et al*, 1989; Capranico *et al*, 1994). At C-9, substitution of the hydroxyl group with a hydrogen atom considerably reduces topoisomerase II-mediated DNA cleavage with concomitant loss of biological activity (Capranico *et al*, 1989). Several studies have

also established structure-activity relationships for the anthraquinones. Drug-induced cleavable complex formation by these compounds has been based on a bipartite structure, in which the chromophore serves as a DNA intercalator and the side chains associate with topoisomerase II, stabilizing the DNA-topoisomerase II interaction (D'Arpa & Liu, 1989; D'Arpa *et al*, 1992). Religation of the DNA strands within the trapped complex is thought to be prevented by the intercalator-induced unwinding of the DNA helix, causing the broken DNA strands to become temporarily misaligned (D'Arpa & Liu, 1989). The topoisomerase II cleavage patterns for ametantrone and mitoxantrone are identical, but ametantrone shows a significantly lower capacity to induce cleavable complexes than mitoxantrone, which suggests a critical role for the 5 and 8 hydroxy substituents of the chromophore in this process (De Isabella *et al*, 1993). This is possibly related to a difference in the orientation of the chromophore within the DNA-drug intercalation site (Mazerski *et al*, 1998; see section 1.2.2).

1.3.2.3 MECHANISMS INVOLVED IN THE CYTOTOXICITY OF TOPOISOMERASE II POISONS

The vast majority of evidence argues that the cytotoxic effects of topoisomerase II poisons such as mitoxantrone and doxorubicin are related to the generation of DNA-topoisomerase II cleavable complexes, or the response to these complexes, rather than inhibition of topoisomerase II catalytic activity *per se* (discussed in detail by D'Arpa & Liu, 1989). However, the exact mechanism by which cleavable complexes are converted to cytotoxic lesions is still under debate. The extent of cleavable complex formation in response to topoisomerase II inhibitors usually reflects enzyme levels, which are highest in the G2/M phases. However, at least for topoisomerase II poisons such as m-AMSA and etoposide, drug treatment is most effective at killing cells during S phase (Wilson & Whitmore, 1981; Chow & Ross, 1987, Estey *et al*, 1987). Consequently, it has long been thought that ongoing DNA synthesis could be one of the elements essential for expression of cytotoxicity. The first direct evidence for this

emerged in 1997, when Catapano and co-workers demonstrated that topoisomerase II-DNA complexes stabilized by the epipodophyllotoxin teniposide acted as replication fork barriers in the c-myc gene of human leukaemia cells. They also found that drug-treated cells were protected from death by the coadministration of the DNA polymerase inhibitor aphidicolin. The latter observation has previously been made by other groups (Schneider *et al*, 1989; D'Arpa *et al*, 1990; Cortes & Pinero, 1994) and a working model for the conversion of topoisomerase II into lethal DNA damage via interaction with replication and transcription forks, proposed by D'Arpa *et al* (1992) is shown in figure 1.9. It is thought that arrest of replication forks may serve as a vehicle for the generation of chromosomal aberrations (Suzuki *et al*, 1995), genetic mutations (Han *et al*, 1993) and G2 phase arrest (Traganos *et al*, 1980) observed in cells treated with topoisomerase II poisons, all of which may promote cell death (Bhalla *et al*, 1993; Ling *et al*, 1993; Bellosillo *et al*, 1998).

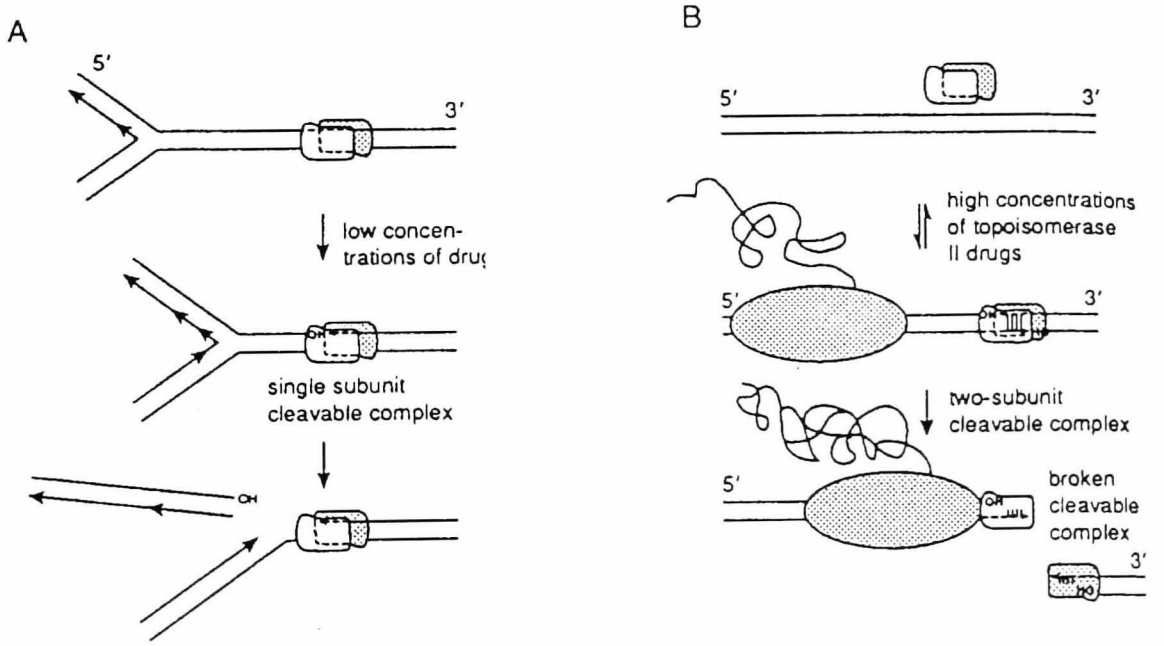


Figure 1.9: Models for the lethal interaction of topoisomerase II cleavable complexes with (A) replication- and (B) transcription machineries. Reproduced from D'arpa *et al*, 1989.

Studies *in vitro* indicate that the cytotoxic activity of topoisomerase II poisons can be mediated by topoisomerase IIα and/or topoisomerase IIβ (Brown *et al*, 1995; Cornarotti *et al*, 1996; Hammonds *et al*, 1998). However, the relative involvement of the α and β isoforms in topoisomerase II poison-mediated cell killing *in vivo* is still unclear. Topoisomerase IIα is expressed at high levels in cycling cells, predominantly during G2/M (Woessner *et al*, 1991), and is the isoform closely associated with chromosome metabolism, including DNA replication (Danks *et al*, 1994; Qiu *et al*, 1996). In contrast, topoisomerase IIβ is expressed at low levels in proliferating cells and is found mainly in the nucleolus (Negri *et al*, 1992; Zini *et al*, 1992). Moreover, *in vivo* studies have shown that topoisomerase IIα activity is elevated during the malignant transformation of ovarian tumours (van der Zee *et al*, 1991, 1994) and that topoisomerase IIα is over-expressed in lung cancer (Giaccone *et al*, 1995) and breast tumours (Murphy *et al*, 1995; Jarvinen *et al*, 1996). All this would suggest that the α isoform is likely to mediate drug sensitivity in the clinical setting. However, many investigators have been unable to find a relationship between either topoisomerase IIα or topoisomerase IIβ levels and sensitivity to topoisomerase II poisons either in cell lines (e.g. Houlbrook *et al*, 1995) or in tumour specimens from patients (e.g. Kaufmann *et al*, 1994). Clearly, a great deal more research is required in this area.

1.3.2.4 DETERMINANTS OF SENSITIVITY TO TOPOISOMERASE II POISONS

A major discrepancy which has come to light in the study of topoisomerase II poisons is the variation in cytotoxic potency for drugs of different classes, even when the initial number of topoisomerase II-mediated DNA strand breaks is the same. Generally speaking, the anthracyclines and mitoxantrone appear to be more potent than compounds such as *m*-AMSA or ellipticine (Binaschi *et al*, 1990; Fox & Smith, 1990). This anomaly may also be partly due to the sequence-specific cleavage of DNA displayed by topoisomerase II poisons (figure 1.10). For example, mitoxantrone has a base preference for cytosine or thymine at the -1 (5') position of the cleavage site

(Capranico *et al*, 1993; De Isabella *et al*, 1993) whereas doxorubicin shows specificity for adenine at this position (Capranico *et al*, 1990). A main determinant of sequence specificity is thought to be the drug pharmacophore, i.e the spatial arrangement and electronic distribution of the drug. Capranico *et al* (1997) have proposed three different types of pharmacophore for topoisomerase II inhibitors, all of which interact with the base pairs from -2 to +2 at the cleavage site (figure 1.11).

In vivo, it could be envisaged that site-selective drug activity would lead to damage of specific genes. In this respect, preferential topoisomerase II-mediated DNA cleavage of c-myc by *m*-AMSA may explain the hypersensitivity of cells that overexpress c-myc such as promyelocytic HL-60 cells, to this agent (Riou *et al*, 1989; Pommier *et al*, 1992).

Since the duration of exposure to cleavable complexes is probably a determinant of cytotoxicity, the prolonged cleavable complex formation observed for mitoxantrone and doxorubicin compared with other agents may also contribute their enhanced potency. Reversal of doxorubicin-induced breaks in small cell lung cancer cell lines is found to be much slower than those produced by ellipticine in two human small cell lung cancer lines (Binaschi *et al*, 1990). Similarly, while mitoxantrone and *m*-AMSA show comparable levels of cleavable complex formation, complexes formed by mitoxantrone are found to be far more persistent and consistently more lethal than those produced by *m*-AMSA (Fox & Smith, 1990; Smith *et al*, 1990). This may be relate to persistent intracellular retention of mitoxantrone, involving slow releasing pools from cytoplasmic inclusions (see section 1.2.2) or different rates of repair of *m*-AMSA and mitoxantrone-induced DNA breaks (Bohr *et al*, 1987; Wassermann, 1994). Alternatively, it may reflect a difference in the mode of DNA-drug binding for mitoxantrone and *m*-AMSA within the ternary complex (Fox & Smith, 1990). Whether this is related to the structure of mitoxantrone *per se* or to one or more active metabolites of the drug is yet to be determined (see section 1.2.3).

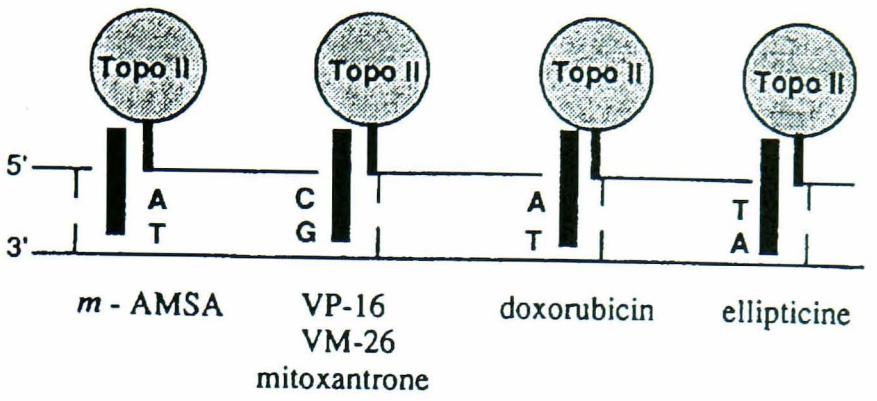


Figure 1.10: Base pair cleavage preferences of various topoisomerase II inhibitors (reproduced from Pommier *et al*, 1994).

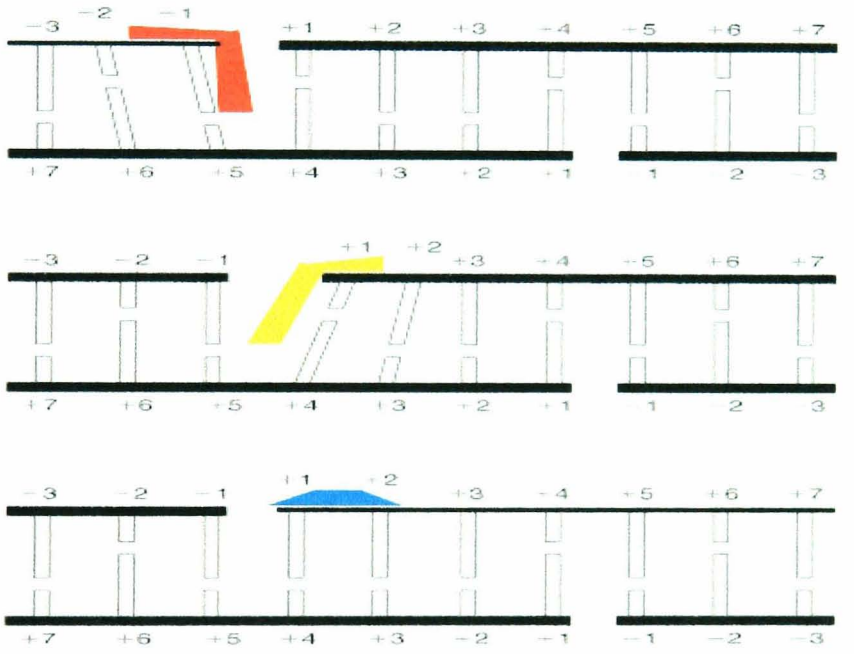


Figure 1.11: Model showing the theoretical interaction of different drug pharmacophores with double stranded DNA (black) within the topoisomerase II cleavage site (representation of the enzyme has been omitted for clarity). The DNA strands are cleaved with a stagger of four base pairs between the two strand cuts. Positive and negative numbers indicate the base numbering on either side of the cleavage site. Poisons with -1 position specificity (red) mainly interact with -1/-2 bases; those with +1 specificity (yellow) interact with +1 base; those with +2 specificity (blue) interact externally with the DNA. Reproduced from Capranico *et al*, 1997.

1.4. PROBLEMS ASSOCIATED WITH CHEMOTHERAPY

1.4.1 DRUG TOXICITY

1.4.1.1 GENERAL EFFECTS

The non-discriminatory nature of conventional anticancer agents such as doxorubicin and mitoxantrone means that as well as acting on cancerous cells, they are also extremely toxic to rapidly proliferating normal tissues. As a result, patients treated with these drugs experience a whole range of undesirable side effects, including nausea and vomiting, gastrointestinal disturbances, radiation recall and severe local reaction following extravasation (see reviews by Posner *et al*, 1985; Abraham *et al*, 1996). Anticancer agents can also produce conditions such as alopecia and discolouration of the skin, which may seem relatively minor, but can be very distressing for the patient. Moreover, both the anthracyclines and mitoxantrone are highly potent in producing malignant transformation and mutation, which presents the risk of secondary malignancies developing some time after the initial treatment (Vallagussa & Bonnadonna, 1995).

1.4.1.2 MAJOR TOXICITIES OF THE ANTHRACYCLINES

One of the most serious side effects encountered when using chemotherapeutic agents is myelosuppression. In the case of the anthracyclines, this is manifest principally as neutropenia (Abraham *et al*, 1996). The condition can be particularly severe with idarubicin, which is more toxic against normal bone marrow progenitor cells than either doxorubicin (Minderman *et al*, 1994) or daunorubicin (Curtis *et al*, 1995). The incidence and severity of immunosuppression is dependent on the drug dose, occurring in 60 to 80% of patients receiving conventional treatment regimens (Abraham *et al*, 1996). As well as affecting absolute numbers of neutrophils, there have also been reports that doxorubicin and daunorubicin reduce the phagocytic function of circulating neutrophils and macrophages (Vaudaux *et al*, 1984). This is highly undesirable situation as it is likely to impair the patient's own immunological defence

against the malignancy.

Haematological toxicity associated with high dose intensity regimens may be partially alleviated by intermittent administration of haemopoietic growth factors in combination with peripheral blood progenitor cells (Crown *et al*, 1992). Alternatively, it may soon be possible to protect haemopoietic progenitor cells from the effects of anticancer drugs by introducing the multidrug resistance gene *MDR1* (Boesen *et al*, 1993: Banerjee *et al*, 1994).

The other major toxicity associated with the use of the anthracyclines is their effect on cardiac tissue, which is manifest as acute, chronic or late-onset cardiotoxicity (reviewed by Shan *et al*, 1996). Mortality directly relating to doxorubicin-induced cardiac failure is substantial, with several reports of death rates above 20%. Various acute and sub-acute cardiotoxic effects of anthracyclines have been documented. These include electrophysiological abnormalities, which may cause non-specific ST and T wave changes, decreased QRS voltage and a prolonged QT interval. The most common rhythm disturbance is sinus tachycardia, but arrhythmias, such as ventricular, supraventricular and junctional tachycardias, have also been reported (Ferrans, 1978). However, electrophysiological alterations such as these are rarely fatal. In clinical terms, the most significant problem is chronic cardiotoxicity, which develops within about a year of therapy. Chronic cardiotoxicity is characterized by irreversible cardiomyopathy, and the incidence of congestive heart failure secondary to anthracycline-induced cardiomyopathy is closely related to the lifetime anthracycline dose. The current recommended cumulative dose of doxorubicin is 550mg/m^2 body surface area. However, the problem with standardizing the dose in this manner is that a wide variation exists in inter-individual sensitivity to anthracyclines. This means that whereas some individuals can tolerate cumulative doses twice as high as those recommended (Bristow *et al*, 1978; Henderson *et al*, 1989), others experience cardiac damage at doses as low as 183mg/m^2 (Friedman *et al*, 1978). Of the many factors proposed to increase chronic anthracycline-induced cardiotoxicity, higher rates of drug

administration, underlying cardiovascular disease and previous cardiac irradiation appear to present most risk (Von Hoff *et al*, 1979; Von Hoff & Layard, 1981).

Contrary to Adamson's hypothesis regarding the cardiac activity of glycosides (see section 1.2.1), the cardiotoxic properties of anthracyclines are currently believed to be mediated by the formation of the semiquinone free radical, generated by one electron reduction of the quinone group of the drug chromophore. Generation of this radical has been demonstrated *in vitro* using NADPH-fortified human liver microsomes (Basra *et al*, 1985) and perfused heart tissue (Doroshow, 1983; Rajagopalan *et al*, 1988). It can also be generated non-enzymically by chelation of the hydroquinone portion of the anthracycline molecule with intracellular ferric iron (Muindi *et al*, 1984). The role of the semiquinone radical in anthracycline cardiotoxicity stems from the fact that it can redox cycle with molecular oxygen to form superoxide anions, which in the presence of metal ions, give rise to highly reactive species such as singlet oxygen, hydrogen peroxide and hydroxyl radicals (Kappus, 1986; figure 1.12). These species are capable of producing non-protein associated DNA strand breaks, but more to the point, they are highly effective in initiating peroxidation of unsaturated membrane lipids, particularly those of the mitochondrial membrane (Goodman & Hochstein, 1977; Mimnaugh *et al*, 1985). The reason why myocardial tissue is particularly susceptible to damage by free radicals is thought to be because of its deficiency in the antioxidant enzymes present in many normal tissues, including catalase, superoxide dismutase and glutathione-S-transferase, which detoxify free radicals and prevent or limit tissue damage (Doroshow *et al*, 1980). However, myocyte death has also been linked to anthracycline-induced intracellular overload of calcium, caused by deregulation of the membrane-bound calcium pump of the sarcoplasmic reticulum (Boucek *et al*, 1987).

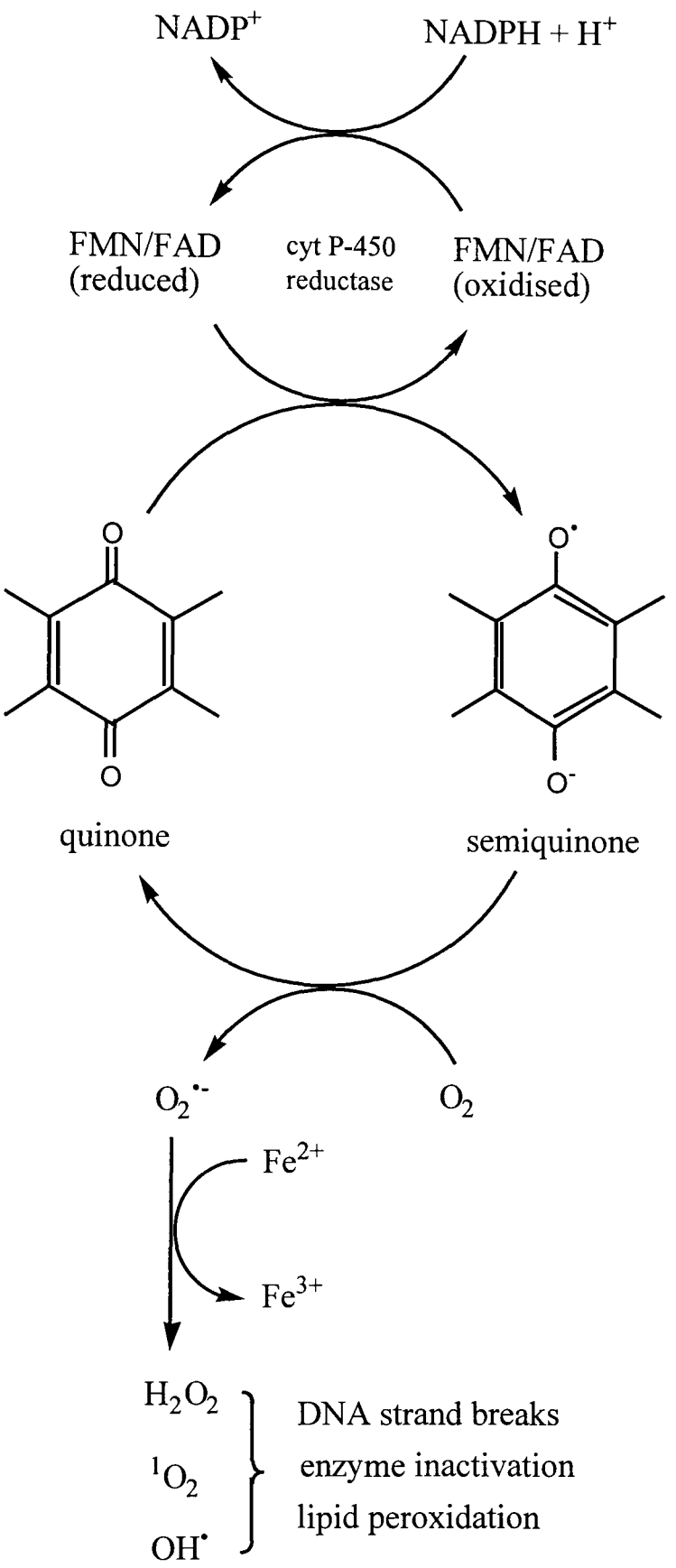


Figure 1.12: Redox cycling of quinone-based anticancer agents to produce reactive oxygen species.

The iron-chelating agent dexrazoxane (ICRF-187) protects against cardiac damage by depleting intracellular iron, thus reducing free radical formation via anthracycline-iron complexes (see Lewis, 1994; Dorr, 1996). In eight comparative clinical studies, this agent reduced the incidence of doxorubicin-induced cardiotoxicity in a total of more than 1500 patients (Hellmann, 1996). However, in a group of patients with breast cancer, combination therapy using doxorubicin, cyclophosphamide and fluorouracil in conjunction with dexrazoxane resulted in lower response rate than patients treated without dexrazoxane (Hortobágyi, 1997).

Various other strategies for reducing anthracycline-induced cardiotoxicity include an administration schedule that produces low peak plasma drug concentrations such as continuous infusion over 48 to 96 hours (Legha *et al*, 1992) or weekly, rather than three- or four-weekly administration (Torti *et al*, 1983). However, there is some concern as to whether drug efficacy is maintained using this approach (Bielack *et al*, 1989). More recently, liposomal formulations have been used to alter the pharmacokinetic properties of anthracyclines. Results to date have demonstrated an improvement in antitumour efficacy using these formulations compared to unencapsulated doxorubicin (Papahadjopoulos *et al*, 1991; Muggia, 1997). Coincidentally, owing to the fact that tumour blood vessels are often leaky, liposomes of 100nm diameter or less are small enough to permeate the tumour vasculature, thereby enhancing drug accumulation in tumours (Yuan *et al*, 1994). Several liposomal anthracycline preparations have been developed, including the polyethylene glycol-coated liposome Doxil (Gabizon *et al*, 1994: Gabizon & Martin, 1997). Unfortunately, a new side effect associated with this preparation is painful dermatitis of the hands and feet, possibly due to deposition of drug in the skin (Gabizon *et al*, 1994).

Several clinical studies have indicated that agents such as epirubicin (Coukell & Faulds, 1997), menogaril (Mazurek *et al*, 1993) and the morpholino anthracyclines (section 1.5.3) are less cardiotoxic than doxorubicin at equivalent doses. The reduced cardiotoxicity of epirubicin is believed to result from the different orientation of the 4'-

hydroxyl group, which is equatorial instead of axial. As a result, when administered *in vivo*, epirubicin is readily detoxified to the 4'-*O*-β-D-glucuronide (Cassinelli *et al*, 1984). Nevertheless, all anthracyclines have cardiotoxic potential, and this remains the major limiting factor associated with their use.

1.4.1.3 TOXIC EFFECTS OF THE ANTHRAQUINONES

In most cases, mitoxantrone displays an improved tolerability profile compared with doxorubicin and other anthracyclines, with respect to the severity of nausea, vomiting and alopecia (reviewed by Shenkenberg & von Hoff, 1986; Faulds *et al*, 1991). However, leucopenia (particularly granulocytopenia) may be dose limiting in patients with solid tumours, and stomatitis may be dose limiting in patients with leukaemia.

Most studies indicate that anthraquinones show insufficient reductive metabolism and redox cycling (Kharash & Novak, 1983; Nguyen & Gutierrez, 1990) and even where reactive oxygen species have been produced (Sinha *et al*, 1983: Basra *et al*, 1985), it has been at levels that do not cause lipid damage (Kharash & Novak, 1982, 1985). In accordance with this, many of the preclinical and earlier clinical studies indicated that mitoxantrone was less cardiotoxic than doxorubicin (see Shenkenberg & von Hoff, 1986; Faulds *et al*, 1991). However, from recent studies, it has become apparent that mitoxantrone poses a significant risk to the cardiovascular system, especially in patients who have pre-existing cardiovascular disease or a history of treatment with anthracyclines or radiotherapy. For example, in a patient with a history of arterial hypertension and previous inferior myocardial infarction, two 20mg doses of mitoxantrone administered four weeks apart caused increasing dyspnea with consecutive left heart failure, pulmonary congestion and lowered blood pressure (Kahles *et al*, 1997). This was accompanied by the appearance of down-sloping ST segments and inverted T waves. Clinical recovery was achieved after six days, but the changes to the electrocardiogram took four weeks to normalise.

The dose of mitoxantrone given in conjunction with other anticancer agents is also critical. In a study by Bowers *et al* (1993) breast cancer patients were treated with increasing amounts of mitoxantrone in conjunction with 900 mgm^{-2} thiotepa, following treatment with 160-492 mgm^{-2} of doxorubicin. When 50 mgm^{-2} mitoxantrone was administered, no acute cardiotoxic effects were observed. In contrast, when 60 mgm^{-2} mitoxantrone was received, congestive heart failure occurred in 4 of the 31 patients. Another patient developed acute pericarditis, and four patients had more than a 10% decline in left ventricular ejection fraction, so that the overall incidence of acute cardiotoxicity at the higher dose was 29%. Although acute cardiotoxicity induced by mitoxantrone is rarely life threatening, mortality has been reported. For example, a 42 year old patient, treated with a cumulative dose of 300mgm^{-2} doxorubicin and 50mgm^{-2} of mitoxantrone, died after being admitted to hospital with rapidly progressive CHF (de Graaf *et al*, 1997). Moreover, she did not receive radiotherapy and had no pre-existing heart disease.

While the propensity of mitoxantrone to undergo reductive metabolism may be relatively low, a recent study of anthraquinone-induced cell injury in cardiomyocytes revealed that mitoxantrone is capable of altering both the membrane integrity and the morphology of these cells (Andersson *et al* ,1999). In fact, the acute toxicity of mitoxantrone in myocytes was found to be considerably greater than either doxorubicin or daunorubicin. Further studies of the metabolism of anthraquinones by cardiac tissue are needed before the full implications of these results are known.

Unfortunately, dexrazoxane has been shown not to afford cardioprotective activity against mitoxantrone (Alderton *et al*, 1992). The use of liposomal preparations of mitoxantrone (Chang *et al*, 1997), mitoxantrone-loaded microspheres (Jameela *et al*, 1996) and mitoxantrone-loaded nanoparticles (Zhang *et al*, 1996) is currently under investigation.

1.4.1.4 DEVELOPMENT OF THE ANTHRAPYRAZOLES

The anthrapyrazole antitumour agents were developed following in vivo studies with the anthracycline 5-iminodaunorubicin, which showed it possessed a reduced cardiotoxic potential compared with other anthracyclines (Tong *et al*, 1979; figure 1.13). Using this principle, the same structural modification was then applied to the anthraquinone chromophore, by substituting one of the quinone carbonyl groups for an imino group. This allowed ring closure with the 2-nitrogen to form a fourth ring system, and thus the anthrapyrazole chromophore was created.

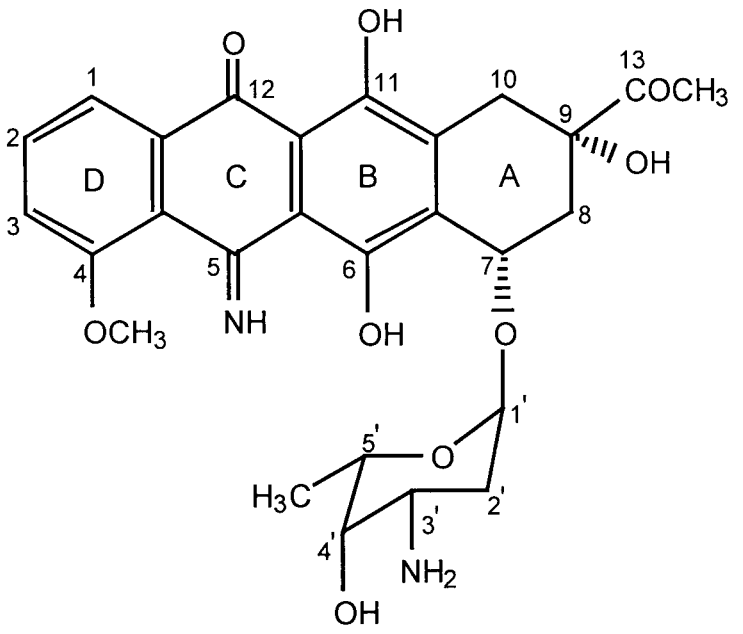
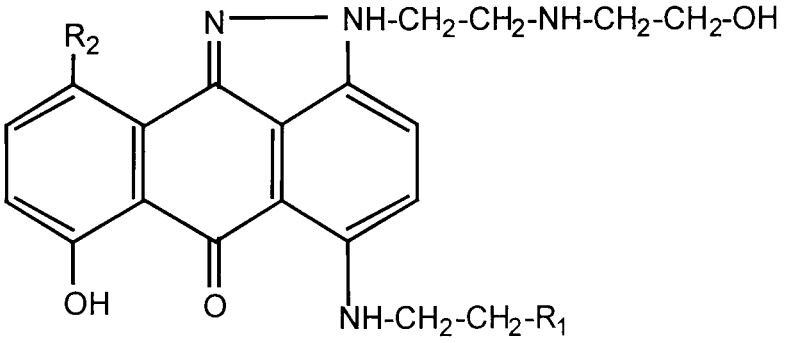


Figure 1.13: The anthracycline 5-iminodaunorubicin

Preclinical studies demonstrated a number of anthrapyrazoles had a similar activity to doxorubicin, and superior activity to mitoxantrone, in terms of both potency and their spectrum of activity (Showalter *et al*, 1987). Among these were CI941 and CI942, which bare close structural resemblance to mitoxantrone (figure 1.14). Like mitoxantrone, CI941 was found to be a potent topoisomerase II inhibitor, generating double stranded DNA breaks by stabilisation of the DNA-topoisomerase II cleavable

complex (Leteurtre *et al*, 1994). Studies employing NADPH fortified liver microsomes and purified cytochrome P-450 reductase showed that not only was CI941 resistant to metabolic reduction, but it actually inhibited doxorubicin stimulated lipid peroxidation (Graham *et al*, 1987). Thus, it was speculated that this anthrapyrazole could have a protective role if used in combination with doxorubicin.

In clinical trials, CI941 demonstrated good clinical efficacy in the treatment of breast cancer (Talbot *et al*, 1991). Unfortunately though, it has subsequently been found to be cardiotoxic (Walsh *et al*, 1995). Among the newer anthrapyrazole analogues are the 9-aza-anthrapyrazoles, which contain a nitrogen atom in the chromophore but lack the chromophore hydroxy groups. A preclinical study carried out by Krapcho *et al* (1998) indicated that these analogues possess minimal or negligible delayed cardiotoxicity, and at least two of the compounds were identified as potential clinical candidates.



CI941: R1=$NH(CH_2)_2OH$; R_2=H
CI942: R1=CH_2NH_2 R_2=OH

Figure 1.14: The anthrapyrazole antitumour agents CI941 and CI942

1.4.2 MULTIDRUG RESISTANCE

Of the many obstacles to the successful treatment of malignant disease, the most difficult to overcome is drug resistance. Some types of cancer (e.g. renal, colon, hepatoma) are inherently resistant to chemotherapy. Others initially respond well to treatment but become increasingly resistant as therapy progresses (see review by Ozols, 1995). This is believed to be due to the presence of small clones of resistant cells in the original tumour which expand by selection during treatment and eventually re-establish the malignancy.

The mechanisms involved in multidrug resistance have been the subject of numerous investigations *in vitro* and to a lesser extent in the clinical setting. The *in vitro* models are provided by cultured cell lines selected for drug resistance by stepwise exposure to a single cytotoxic agent over a long period of time (Schoenlein, 1993). These lines often display multiple resistance mechanisms and develop cross-resistance to a wide variety of structurally and functionally unrelated cytotoxic agents, a phenomenon known as multidrug resistance (MDR). Exposure of cultured cells to topoisomerase II inhibitors usually induces drug efflux-mediated resistance mechanisms, or alterations to the target enzyme itself.

1.4.2.1 DRUG EFFLUX-MEDIATED MULTIDRUG RESISTANCE

The archetypal mechanism of resistance to anticancer agents is mediated by P-glycoprotein, a 170KDa plasma membrane-bound protein belonging to the ATP-binding cassette (ABC) superfamily of transporter systems (Higgins, 1992). P-gp acts as a unidirectional, energy-dependent drug efflux pump, thereby reducing intracellular drug concentrations to a tolerable level (Endicott & Ling, 1989; Gottesman & Pastan, 1993). Cancer cells which overexpress P-glycoprotein are said to manifest the classical MDR phenotype, characterized by cross-resistance to drugs such as the anthracyclines, anthraquinones, vinca alkaloids and epipodophyllotoxins, but not to alkylating agents, antimetabolites or cisplatin (Nooter & Stoter, 1996). P-glycoprotein is also found in

normal tissues including the liver, small intestine and kidneys (Thiebaut *et al*, 1987) where it is thought to have physiological roles in the elimination of xenobiotics and transport of steroid hormones (Borst *et al*, 1993). Thus it is hardly surprising that the malignancies resulting from these tissues often express the highest levels of P-gp (Goldstein *et al*, 1989).

Elevated levels of P-gp are commonly due to up-regulation of the *MDR1* gene located on human chromosome 7. Direct proof that the *MDR1* gene is responsible for P-gp-mediated MDR has been obtained by transfection of a full-length cDNA clone of the human *MDR1* gene into a drug sensitive cell line, which thereafter became drug resistant (Ueda *et al*, 1987). However, P-gp expression can also be regulated through stabilization of P-gp mRNA, or via post-translational stabilization mechanisms (McClean & Hill, 1993). Also in cell lines, expression of P-gp can be induced by antitumour drugs (Hu *et al*, 1993). This may be one of the reasons why tumour cells that are initially sensitive to treatment become resistant after repeated exposure to these agents. It could also be of clinical significance because chemotherapy often fails due to the survival of a relatively small number of drug resistant cells in the original tumour, which subsequently proliferate to form a far more aggressive malignancy that is unresponsive to chemotherapy.

To some extent, P-gp-mediated resistance can be overcome by intensifying the drug dose. However, this almost invariably leads to reduced tolerability. Numerous anthracycline analogues have been developed in an attempt to circumvent drug resistance associated with doxorubicin and daunorubicin, including the morpholino anthracyclines (see section 1.5.3), iododoxorubicin (Schott *et al*, 1990) and idarubicin (see section 1.2.1). These analogues are more lipophilic than the parent compounds, which improves their intracellular uptake and accumulation, and hence their cytotoxic activity (Fukushima *et al*, 1993; Smith *et al*, 1994a). Also, since the discovery that the calcium channel blocker verapamil can reverse classical MDR, much research has focused on the development of chemical modulators of P-gp (reviewed by Beck, 1990;

see table 1.1). Many of these agents possess a planar aromatic ring system with two or more rings, a tertiary nitrogen and a positive charge at physiological pH and appear to overcome resistance by competing with drugs for binding to P-gp (Zamora *et al*, 1988).

Table 1.1 Modulators of P-gp mediated multidrug resistance*

Type of modulator	Example
Detergents	cremophore EL
Calcium channel blockers	phenylalkylamines (e.g verapamil); dihydropyridines
Calmodulin inhibitors	phenothiazines
Coronary vasodilators	dipyridamole, perhexiline, amiodarone
Indole alkaloids	reserpine
Quinolines	chloroquine, quinine
Acridines	quinacrine
Lysomotropic agents	monensin, nigericin
Steroids	progesterone
Triparanol analogues	tamoxifen, clomiphene
Cephalosporins	cefoperazone, ceftriaxone
Cyclosporins	cyclosporins A, C, G

* Reproduced from Beck, 1990.

Verapamil has been found to reverse P-gp-mediated doxorubicin resistance in several human cell lines including ovarian cancer cells (Rogan *et al*, 1984) and a multiple myeloma cell line (Bellamy *et al*, 1988). However, it has less effect on idarubicin (Boiron *et al*, 1994) and has little effect on the cytotoxicity of mitoxantrone (Gibby *et al*, 1987). Moreover, mitoxantrone is only partially cross-resistant with doxorubicin in the P-gp-positive cell line P388/Dx (Testi *et al*, 1995). These observations indicate that resistance to mitoxantrone in some cell lines may be mediated by a drug efflux mechanism discrete from P-gp. Indeed, in 1992, Cole and

her colleagues discovered a novel type of multidrug resistance phenotype, characterized by overexpression of the 190kDa multidrug resistance associated protein. MRP (reviewed by Kavallaris, 1997). Rather like P-gp, MRP is a member of the ABC superfamily, which acts as a drug efflux pump and is located primarily in the plasma membrane. However, it has also been speculated that it may promote drug accumulation in specific compartments within the cell in order to aid efflux or limit the interaction of drugs with intracellular target molecules (Almquist *et al*, 1995). MRP can also mediate resistance by promoting the export of glutathione-conjugated drugs from cells and therefore has a connection with drug resistance mechanisms involving glutathione and glutathione-related enzymes (see section 1.4.2.4). Broadly speaking, MRP displays cross-resistance to a similar range of cytotoxic agents to P-gp (Nooter & Stoter, 1996), but their susceptibility to reversing agents differs considerably. Chemical modulators of MRP include genestein (Versantvoort *et al*, 1993) and difloxacin (Gollapudi *et al*, 1995).

1.4.2.2 CLINICAL EVALUATION OF P-GLYCOPROTEIN- AND MRP-MEDIATED DRUG RESISTANCE

While P-gp-mediated multidrug resistance has been extensively characterized *in vitro*, relatively few studies have been performed to evaluate its clinical significance. The expression of *MDR1* has been detected in a wide range of malignancies, either treated or untreated (Nooter & Stoter, 1996). However, this seems to be of prognostic value in relatively few types of cancer (table 1.2).

Presently, information on the clinical role of MRP expression is relatively sparse. Although elevated MRP expression has been reported in haematological malignancies, NSCLC, and cancers of the breast and ovary (Nooter & Stoter, 1996), the association between MRP and clinical resistance has yet to be determined. Preliminary evidence indicates that in patients with non-small-cell lung cancer, MRP overexpression of the tumours predicted a worse outcome of vindesine/etoposide chemotherapy (Ota *et al*,

1995). The absence of MRP expression was also suggested to be the reason for good outcome of an AML subtype (Kuss *et al*, 1994). Just to confuse matters, it has recently been reported that the expression of another MDR-associated transporter protein, the 110kDa LRP, predicted clinical outcome in AML (List *et al*, 1996) and advanced ovarian carcinoma (Izquierdo *et al*, 1995).

Table 1.2 Types of malignancy in which *MDR1*/P-gp have prognostic value

AML
ALL
Childhood soft tissue sarcoma
Neuroblastoma
Osteosarcoma
Breast cancer
Non-Hodgkin's lymphomas
Multiple myeloma

Although modest reversal of drug resistance has been achieved in the clinic employing verapamil, its use is limited by its adverse effects on the cardio-vascular system (Miller *et al*, 1991). The immunosuppressant cyclosporin A has also been employed in clinical trials, but again displays a variety of toxic side-effects (Sonneveld *et al*, 1992). Second generation analogues of both these chemical modulators are now under clinical investigation, including the D- stereoisomer of verapamil and the cyclosporin analogue PSC 833 (Ozols, 1995). However, as clinical MDR is generally multifactorial, the presence of non-P-glycoprotein mediated drug resistance mechanisms insensitive to these compounds may limit the success of such treatment strategies.

1.4.2.3 THE AT-MDR PHENOTYPE

Cells which are only cross-resistant to topoisomerase II-inhibiting drugs and show no alterations in drug transport or accumulation are said to display atypical multidrug resistance (at-MDR). Cell lines selected for at-MDR are resistant to DNA intercalating agents and epipodophyllotoxins, but unlike the classical MDR- and non-P-gp phenotypes, are not cross-resistance with vinca alkaloids (Nooter & Stoter, 1996).

The at-MDR phenotype is characterized by a reduction in topoisomerase II levels or changes that alter the interaction of the enzyme with drug or DNA. In addition, cells such those in hypoxic tumour regions are inherently resistant to topoisomerase inhibitors as they are non-cycling (see section 1.5.4.1). Resistant cells use a multitude of strategies to impair enzyme-DNA or -drug interactions, several of which may operate simultaneously (table 1.3). These strategies aim to achieve a reduction in the level of drug-induced DNA cleavage without overall loss of catalytic activity. For example, HL-60/MX2, a human leukaemia cell line selected for resistance to mitoxantrone, has been found to contain a novel 160KDa topoisomerase IIα-related protein localized in the cytoplasm, which is thought to be missing the nuclear recognition signals located in the COOH terminus of the protein (Harker *et al*, 1995). Although the 160KDa protein retains catalytic activity, cytosolic localization may preclude its involvement in the important drug-DNA-enzyme interactions necessary for DNA cleavage and eventual cell death. In addition, cytoplasmic topoisomerase II may act as a drug sump which prevents or delays drug entry into the nucleus. This is supported by the fact that mitoxantrone-induced DNA cleavage in the HL-60/MX2 nuclear extract is reduced 3-4-fold compared to the parental HL-60 cells (Harker *et al*, 1991).

Enzyme mutations also appear to be a common feature of topoisomerase II-mediated resistance *in vitro*. Point mutations in the gene for topoisomerase IIα have been observed in cultured cell lines selected for resistance to intercalating agents, epipodophyllotoxins or both (reviewed by Prost, 1995). The majority of these are

located around the consensus B sequence of the ATP binding fold and the dinucleotide-binding βαβ unit, or near the reactive tyrosine residue. This is not surprising in view of the fact that these regions are involved in ATP-, DNA- or drug binding to the enzyme. Having said that, mutations can also alter the physiological properties of the enzyme, such as the pH optimum for catalytic activity (Boege *et al*, 1993).

Table 1.3 Summary of phenotypic alterations displayed by at-MDR cells

reduction in topoisomerase II levels
alterations in the proportion of topoisomerase IIα and/or β
enzyme mutation
post-translational modification of topoisomerase II (e.g. phosphorylation)
alterations in subcellular distribution of topoisomerase IIα

It is known that non-cycling cells, which contain low levels of topoisomerase IIα, lack sensitivity to topoisomerase inhibitors (see section 1.3.2.3). This would suggest that this isoform may play a predominant role in the action of these agents in the clinical environment. Moreover, the use of genetic suppressor elements to decrease the α isoform was shown to result in expression of resistance (Hill, 1996). However, the observation that topoisomerase IIβ was undetectable in the HL-60/MX2 model mentioned above and three other mitoxantrone resistant cell lines has prompted speculation that the β isoenzyme may be involved in resistance to mitoxantrone (Harker *et al*, 1995; Withoff *et al*, 1996). In addition, VP-16-resistant H209/V6 cells, that contain no normal topoisomerase IIα enzyme but display topoisomerase IIβ enzyme levels that are comparable to the H209 parent, are not cross-resistant to mitoxantrone (Mirski *et al*, 1993; Feldhoff *et al*, 1994).

Very few mutations have been found in patients' tumours and this would seem to indicate that clinical resistance is primarily due to a reduction in topoisomerase II expression (e.g. see Potesmil *et al*, 1988). However, two point mutations have been

found in a SCLC lung cancer patient at nucleotide positions 1457 and 1481 (Kubo *et al*, 1995). Also, in an AML patient, the argenine residue at amino acid position 773 of topoisomerase IIα was replaced by isoleucine (Danks *et al*, 1993). The roles of the two topoisomerase II isoforms in clinical drug resistance is relatively sparse and studies in this area are currently underway (see Holden, 1997). Another issue that needs to be resolved is whether the bulk topoisomerase II content in a patient's tumour can be correlated with sensitivity of the tumour to drug treatment. This has been difficult to demonstrate due to the large cell-to-cell topoisomerase IIα expression within tumour samples, which is thought to result from the presence of large numbers of G0 phase-arrested cells (Kaufmann *et al*, 1994; Stammler *et al*, 1994).

In general, chemical modulators of P-gp- and MRP-mediated resistance can not be used in cells expressing only at-MDR. One exception is the P-gp inhibitor dipyridamole, which significantly potentiates the cytotoxicity of mitoxantrone, doxorubicin and etoposide in multidrug resistant melanoma cells (Damle & Desai, 1994). As the morpholino anthracyclines act by a mechanism which is largely unrelated to topoisomerase II, they can also circumvent resistance in at-MDR cells (see section 1.5.3) as can some aminoacridine analogues (Baguley *et al*, 1990). The potential for using inhibition of protein kinases to overcome resistance by controlling the phosphorylation status of topoisomerase II (and P-gp) is also under investigation (Grunicke *et al*, 1994).

1.4.2.4 OTHER MECHANISMS OF RESISTANCE TO TOPOISOMERASE II INHIBITORS

Several other mechanisms of resistance to mitoxantrone have been observed *in vitro*. These include an alteration in the expression of cytokeratin networks in mouse L fibroblasts (Bauman *et al*, 1994) and altered cytoplasmic mitoxantrone sequestration in drug resistant murine cell lines (Smith *et al*, 1992) and human colon carcinoma cells (Fox & Smith, 1995). There is also evidence for reduced drug accumulation in

association with drug resistance to mitoxantrone in MCF-7 human breast cancer cells without associated P-glycoprotein or MRP expression (Lee *et al*, 1997)

Elevated expression and activity of glutathione-peroxidase, an enzyme important in the detoxification of lipid peroxides and free radicals, has been shown in several doxorubicin-resistant cell lines, suggesting it may contribute to drug resistance in these cells (see review by Zhang *et al*, 1998). The use of the glutathione synthesis inhibitor buthionine sulfoximine (BSO) was found to partially reverse resistance to daunorubicin in HL-60/AR anthracycline resistant human leukaemia cells (Lutzky *et al*, 1989). The development of similar agents which could be used for this purpose may be an area worth exploring.

Topoisomerase II inhibitors are known to induce programmed cell death (see section 1.3.2.3) and a decrease in apoptosis has already been reported in numerous cell lines resistant to topoisomerase poisons, including an AML cell sub-line which displays natural resistance to mitoxantrone (Bailly *et al*, 1997). It is likely that many other resistance mechanisms will be discovered in both cell lines and *in vivo*. Wherever possible, the resistance profiles of clinical tumours should be assessed on an individual basis, because this is likely to provide the best chance of successful treatment.

1.5 THE USE OF METABOLICALLY ACTIVATED ANTITUMOUR AGENTS IN THE TREATMENT OF CANCER

1.5.1 ENZYMES INVOLVED IN DRUG METABOLISM

Upon entering the body, therapeutic agents undergo a series of metabolic transformations, mainly in the liver. Phase I (functionalization) reactions, as their name suggests, serve to introduce a reactive functional group into the molecule thereby converting the drug into a more polar form. Common phase I reactions include oxidation, reduction, hydrolysis and hydration (see Gibson & Skett, 1986). The products of phase I reactions often go on to be used as substrates for phase II

(conjugation) reactions such as glucuronidation, sulfation, acetylation and glutathione conjugation. These are widely considered as the true detoxification pathways as they give rise to the bulk of the inactive drug, which is then excreted.

In vitro, the majority of knowledge regarding drug metabolism has been obtained using subcellular fractions of the liver. The two subcellular organelles which are the most important in this respect are the endoplasmic reticulum and the cytosol (soluble fraction). Phase I oxidative enzymes are almost exclusively located in the endoplasmic reticulum, along with the phase II enzyme glucuronyl transferase. Other phase II enzymes are found predominantly in the cytosol (Lebsanft *et al*, 1989). In the intact cell, endoplasmic reticulum consists of a continuous network of filamentous membrane-bound channels (Alberts *et al*, 1983). Physical disruption of endoplasmic reticulum results in the formation of artificial vesicles, operationally described as microsomes, which can be separated from the cytosolic fraction by consecutive differential centrifugation.

The most extensively studied phase I drug metabolism reaction is cytochrome P-450-mediated mixed function oxidation, which catalyses the hydroxylation of hundreds of structurally diverse compounds (see Gibson & Skett, 1986). Cytochrome P-450 is a haemoprotein, with iron protoporphoryn IX as the prosthetic group. The central feature of the cytochrome P-450 catalytic cycle is the ability of the haem iron to undergo cyclic oxidation/reduction reactions in conjunction with the associated flavoprotein reductase, NADPH-cytochrome P-450 reductase (figure 1.15). The end result is the incorporation of one atom of molecular oxygen into the substrate and the reduction of the other oxygen atom to water.

So far, more than 20 different human isoenzymes of cytochrome P-450 have been identified, and divided into four main families (1-4) and further into sub-families (A-E) according to their amino acid homologies (Nelson *et al*, 1993). Of the human cytochrome P-450 enzymes, CYP3A accounts for approximately 60% of the total P-450 and CYP3A4 is the predominant isoform associated with the metabolism of

chemotherapeutic agents (Guengerich, 1992; Spatzenegger & Jaeger, 1995). Although each isoform has a particular substrate specificity, the existence of multiple P-450 isoforms means that the range of drugs susceptible to cytochrome P-450 phase I biotransformation is diverse. As well as varying from species to species, there is also a large interindividual variability in cytochrome P-450 expression, as the genes encoding many of these enzymes are polymorphically expressed (Wrighton *et al*, 1989; Smith *et al*, 1994b). This largely accounts for differences in drug metabolism (and perhaps clinical response) observed among patients who are given identical treatment (Cholerton *et al*, 1992).

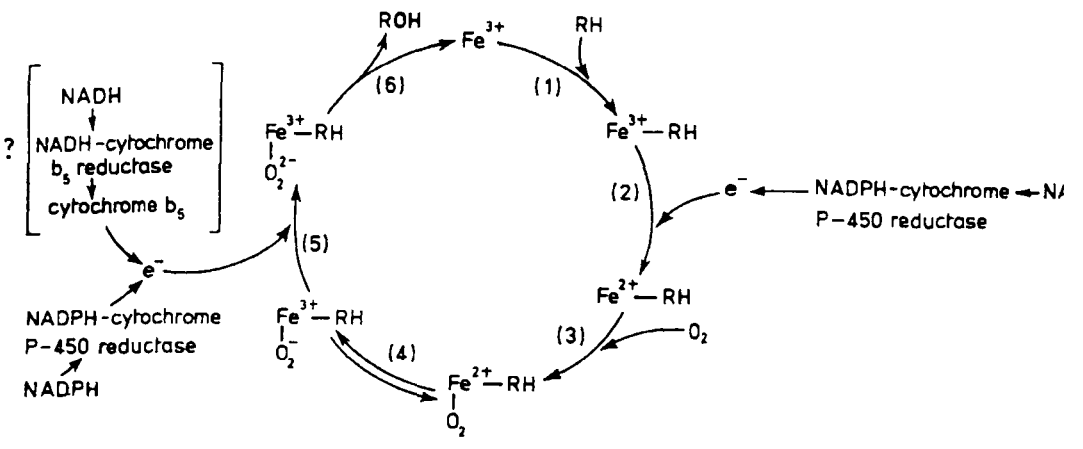


Figure 1.15: The catalytic cycle of cytochrome P-450 (reproduced from Gibson & Skett, 1986). RH represents the drug substrate, ROH the corresponding hydroxylated metabolite.

In order to determine the relative contribution of the various isoforms to the metabolism of a particular drug, the expression of specific cytochrome P-450 isoforms (and non-P-450 drug metabolizing enzymes) can be enhanced by various chemical inducers, well known examples being dexamethazone and phenobarbital (Spatzenegger & Jaeger, 1995). For similar purposes, cytochrome P-450 isoenzymes can also be inhibited. For example, cyclosporin A and erythromycin are both competitive substrates of P-450 3A (Kronbach *et al*, 1988) whereas carbon monoxide is a general

inhibitor of haemoproteins (Gibson & Skett, 1986).

So far, cytochrome P-450 enzymes have been implicated in the oxidative activation of a broad range of anticancer agents, including etoposide (Van Maanen *et al*, 1985a, 1985b, 1987), cyclophosphamide (see section 1.5.2) and the morpholino anthracyclines (see section 1.5.3). However, they are by no means the only phase I enzymes involved in drug oxidation. Among the others are flavin monooxygenases. various oxidases (e.g. aldehyde-, xanthine- and monoamine oxidases) and dehydrogenases (see Beedham, 1997). NAD-dependent alcohol- and aldehyde dehydrogenases are mitochondrial and cytosolic enzymes which sequentially oxidize compounds containing primary alcohols to carboxylic acids via their aldehyde intermediates (Bosron & Li, 1980; Pietrusko *et al*, 1991). Metabolism is usually extremely rapid, so that only the carboxylic acid metabolites are detected. Carboxylic acids are major metabolites for a number of anti-cancer drugs, including cyclophosphamide and mitoxantrone (see sections 1.2.3 and 1.5.2.2).

Expression of cytochrome P-450 and other drug-metabolizing enzymes in tumour tissues may be different from that in normal tissues. Toussaint *et al* (1993) showed that cytochrome P-450 1A1/1A2 levels in small cell lung cancer cells were three-fold lower than in the corresponding non-tumour cell samples. Similarly, El Mouelhi *et al* (1987) found significant reductions in both phase I and II enzymes in primary hepatic carcinomas, compared with normal hepatic tissue. Conversely, the bioreductive enzyme DT-diaphorase was reported to be expressed at high levels in some human tumours of the breast, lung liver and colon (Riley & Workman, 1992). Moreover, expression of the cytochrome P-450 3A isoform has been detected in human malignancies, such as colon, oesophagus and bladder, where the level of CYP3A in the corresponding normal tissues was undetectable (McKay *et al*, 1993; Murray *et al*, 1994, 1995). Recently, CYP1B1 has been identified as a human tumour specific cytochrome P-450 isoform (Murray *et al*, 1997). Obviously, the qualitative and quantitative expression of drug-metabolizing enzymes in tumour tissues will play a

major part in determining the degree of drug metabolism in a particular tumour and hence the antitumour efficacy of the drug (see Cholerton *et al*, 1992).

1.5.2 PRODRUGS

1.5.2.1 PRODRUG STRATEGIES

In contrast to serving as a means of pharmacological deactivation of a drug, in certain cases, biotransformation can result in an increase in the expression of pharmacological activity. This can be exploited in the treatment of cancer by designing pharmacologically inert compounds known as 'prodrugs', which are selectively activated in the body, either by chemical means or metabolism (reviewed by Denny, 1996). With respect to cancer treatment, the ideal prodrug is one which is activated specifically in the tumour cell environment rather than in all tissues. This reduces the systemic toxicity associated with chemotherapy, which in turn should allow the administration of higher concentrations of drug, with a better chance of achieving tumour response. Obviously, to achieve selective activation, the tumour cell target must possess a functional characteristic not shared by normal cells. In this respect, several new techniques have emerged over recent years, including antibody- and genetic-based methods for targeting activating enzymes to tumours in a specific manner. Gene-directed enzyme prodrug therapy (G-DEPT) involves targeting the activating enzyme to the tumour cell by incorporating the gene encoding the enzyme into the genome of the tumour cell using a suitable vector, usually a liposome or viral carrier (Denny & Wilson, 1998). Antibody-directed enzyme prodrug therapy (A-DEPT), on the other hand, works by introducing a foreign enzyme to tumour cells by linking it to monoclonal antibodies raised against tumour specific antigens. This is followed by administration of the prodrug which is activated specifically by the enzyme.

1.5.2.2 CYCLOPHOSPHAMIDE; THE ARCHETYPAL ANTICANCER PRODRUG

One of the most widely used prodrugs in the clinic today is the DNA alkylating agent cyclophosphamide, which has a high therapeutic index and a broad spectrum of activity against a variety of haematological malignancies and solid tumours (Friedman *et al*, 1979). The selectivity of cyclophosphamide towards cancer cells stems from the two alternative routes of biotransformation of its initial metabolite (figure 1.16), one leading to pharmacological activation, the other to deactivation (Cox *et al*, 1976a).

Cyclophosphamide is initially oxidized by cytochrome P-450 to 4-hydroxy-cyclophosphamide which exists in equilibrium with an alicyclic aldehyde, aldophosphamide. Various studies have shown that members of the CYP2B, CYP2C and CYP3A subfamilies are all effective in catalysing this reaction (Spatzenegger & Jaeger, 1995). Under relatively anaerobic conditions in tumour cells, the aldehyde spontaneously decomposes to acrolein and phosphoramide mustard. Although acrolein can bind covalently to proteins (Marinello *et al*, 1984; Ohno & Ormstad, 1985), the primary cytotoxic metabolite is almost certainly the phosphoramide mustard, which spontaneously decomposes to produce a positively charged aziridinium group, with the loss of chloride (Williamson & Witten, 1967). This reacts with DNA, predominantly with the N7 positions of guanine residues, in an S_N2 alkylation step (Kohn *et al*, 1987).

As the reaction between the drug and DNA is covalent, DNA damage by cyclophosphamide is potentially more lethal than damage produced by agents that bind to DNA non-covalently, such as DNA intercalators. This is due to the fact that the lesions formed are far more persistent and cells must undergo extensive DNA repair to remove them (see reviews by Wassermann, 1994; Chaney & Sancar, 1996). In addition, being a bis(2-chloroethyl)amine, phosphoramide mustard contains two alkylating groups on each drug molecule, enabling it to cross-link nucleophilic atoms in nucleic acids, proteins or other biomolecules (Lawley & Brookes, 1967). Indeed, the effective nitrogen mustards are almost universally bifunctional, suggesting that their anticancer action involves the formation of cross-links between macromolecular sites.

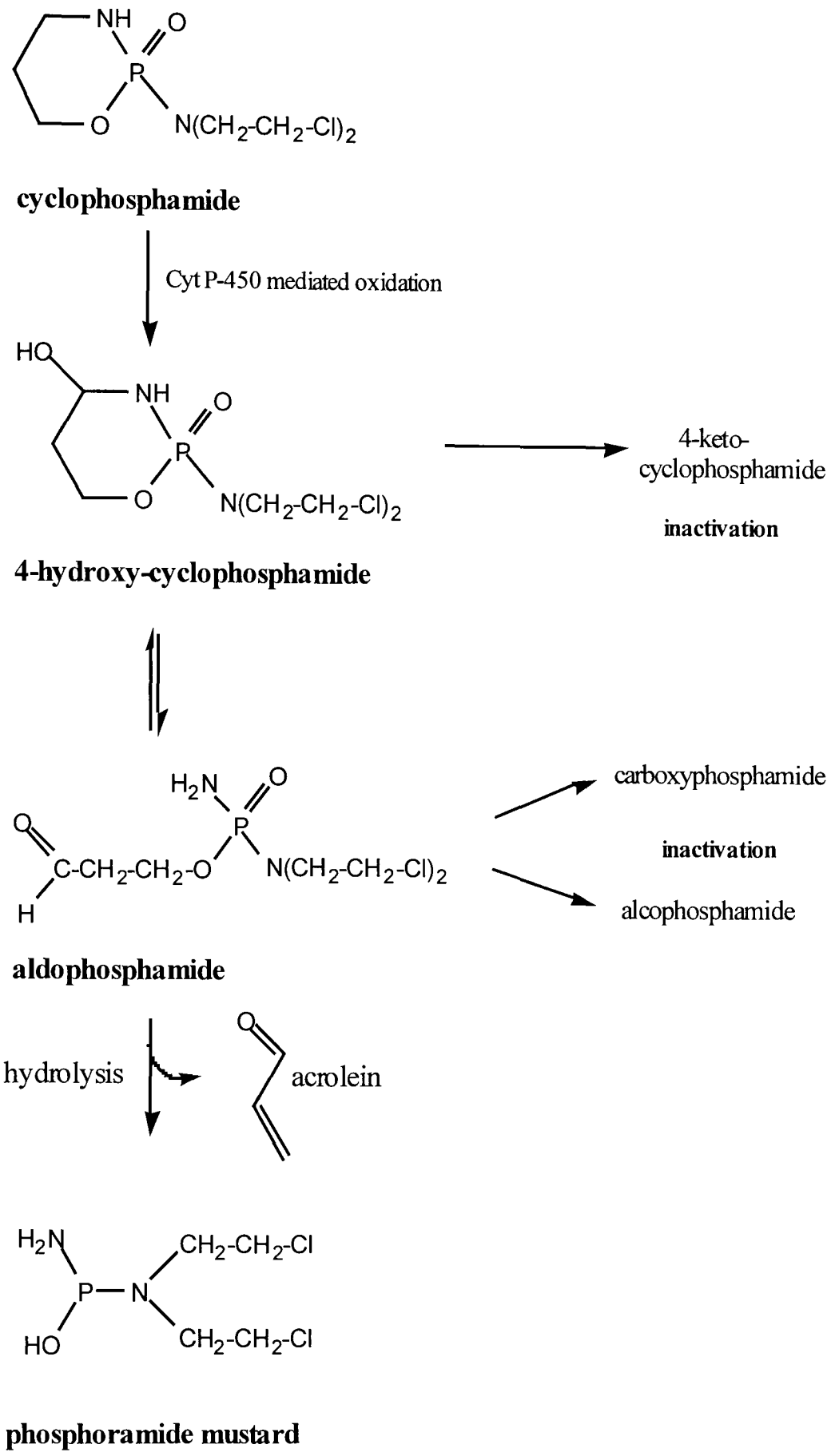


Figure 1.16: Metabolic activation of cyclophosphamide to produce the active phosphoramide mustard (adapted from Gibson & Skett, 1986).

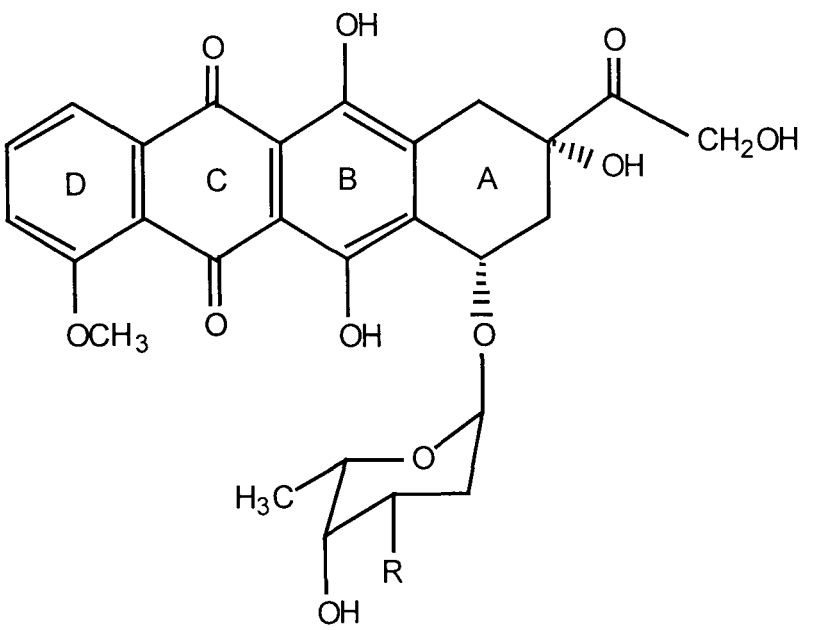
Perhaps the most important alkylation process is DNA-DNA interstrand cross-linking, which inhibits DNA strand separation, and can thus constitute a complete block to DNA replication and transcription (Gralla *et al*, 1987; Pieper *et al*, 1989). The inability of complementary strands of cross-linked DNA to separate has been demonstrated experimentally (Kohn *et al*, 1966) and both interstrand cross-links (Ewig & Kohn, 1977) and DNA-protein cross-links (Thomas *et al*, 1978) have been observed in intact cells.

Cyclophosphamide also undergoes several other biotransformations leading to pharmacological deactivation. By far the most important of these is the oxidation of aldophosphamide to the inactive metabolite carboxyphosphamide by cytosolic aldehyde dehydrogenase isoenzymes (Cox *et al*, 1976b). This appears to be the key determinant in the selective toxicity of cyclophosphamide toward tumour cells, which are usually deficient in aldehyde dehydrogenase activity (Sladek, 1987). However, because oxidation of aldophosphamide is a detoxification reaction, elevated levels of class III aldehyde dehydrogenase levels in tumour cells can lead to acquired resistance to cyclophosphamide (Rekha *et al*, 1994).

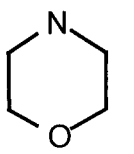
1.5.3 METABOLICALLY ACTIVATED ANTHRACYCLINES; THE MORPHOLINO ANALOGUES

In an attempt to circumvent the toxic side effects and drug resistance associated with the use of the anthracyclines, a series of analogues have been synthesized in which the amino nitrogen at the 3' position of the daunosamine sugar is incorporated into a morpholino ring (see Acton *et al*, 1984). These compounds, known as the morpholino anthracyclines, differ from conventional anthracyclines particularly in their mechanisms of action, cytotoxic potency and activity against *MDR1*-expressing doxorubicin-resistant cell lines. With respect to metabolic transformation, the two most important members of this group are morpholino doxorubicin (mdox) and methoxymorpholino doxorubicin (mmdox) (figure 1.17). *In vitro*, these compounds

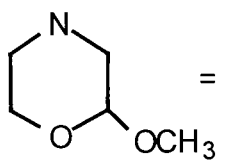
have cytotoxic activity comparable to doxorubicin (Jesson *et al*, 1987, Grandi *et al*, 1990). However, when administered *in vivo*, their potency is far greater than that of doxorubicin (Acton *et al*, 1984, Ripamonti *et al*, 1992), suggesting that the parent compounds are biotransformed *in situ* to highly cytotoxic metabolites.



R = NH_2 = doxorubicin



= morpholino doxorubicin



= methoxymorpholino doxorubicin

Figure 1.17: Structural modification of doxorubicin to produce morpholino- and methoxymorpholino doxorubicin.

1.5.3.1 METABOLIC ACTIVATION OF MORPHOLINO DOXORUBICIN

The effects of mdox *in vivo* can be emulated *in vitro* using microsomal enzyme systems. Incubation of the drug with NADPH-fortified human liver microsomes prior to addition to human ovarian carcinoma cell line ES-2 potentiates its cytotoxicity 44-fold (Lau *et al*, 1989). The active species generated from metabolism of mdox is a potent DNA alkylator, capable of producing interstrand DNA cross-linking. The mechanism for biotransformation of mdox is known to involve hepatic mixed function oxidation. Metabolic potentiation and alkylating activity are abolished by the cytochrome P-450 3A-specific antibody and inhibitors of cytochrome P-450 3A, demonstrating this sub-family plays a vital role in the activation process, probably the CYP3A4 isoform in particular (Lewis *et al*, 1992). Cytotoxic potentiation is substrate specific, with mdox activation being one hundred-fold compared to one or two fold for its closely related analogues morpholino daunorubicin and morpholino oxaunomycin (Lewis *et al*, 1992).

As yet, the active metabolite of mdox has not been identified, but it appears to cross-link DNA by a similar mechanism to that of the closely related analogue cyanomorpholino doxorubicin, which forms intra- and inter-strand cross-links without bioactivation (Begleiter & Johnston, 1985; Jesson *et al*, 1989). Structure-activity studies with several morpholino anthracyclines have indicated that the reactive sites involved in the DNA cross-linking process are the *N*-α carbon atoms located in morpholino ring (Lau *et al*, 1989; Westendorf *et al*, 1989). Acton *et al* (1988) have postulated that mdox is α-hydroxylated to a carbinolamine with subsequent conversion to a reactive iminium ion, which is capable of alkylating DNA. However, the nature of second alkylating substituent involved in cross-linking has not been speculated upon.

1.5.3.2 METABOLIC ACTIVATION OF METHOXYMORPHOLINO DOXORUBICIN

Incubation of mmdox with NADPH-fortified human or murine liver microsomes potentiates its cytotoxic potency 50-fold in the ES-2 cell line (Lau *et al*, 1994a). Similar to mdox, cytotoxic potentiation is associated with the formation of a covalently reactive molecule, capable of forming DNA interstrand cross-links. Again, cytochrome P-450, principally the CYP 3A sub-family, appears to play a major role in the biotransformation process, since potentiation is inhibited by cyclosporin A (Lau *et al*, 1994a) and erythromycin (Lau *et al*, 1991).

The covalently reactive metabolite of mmdox is thought to be the *O*-demethyl derivative produced by oxidation of the β-carbon atom of the morpholino ring (Lau *et al*, 1991). Particularly under acidic conditions, the hemiacetal generated by *O*-demethylation of the β-carbon atom could ring open to form an aldehyde (Fessenden & Fessenden, 1986). This may well be one of the species responsible for the observed DNA cross-linking, as aldehydes are known to react with nucleophilic groups such as those found in proteins and DNA to form Schiff's bases (Rawn, 1983; Fessenden & Fessenden, 1986). However, the exact mechanism of cross-linking has yet to be determined. DNA-drug adducts following metabolism have been detected by gel electrophoresis and HPLC (Graham *et al*, 1992) and structural elucidation of the active metabolite is still under investigation. Possible metabolites of mdox and mmdox with the potential for alkylating DNA are shown in figure 1.18.

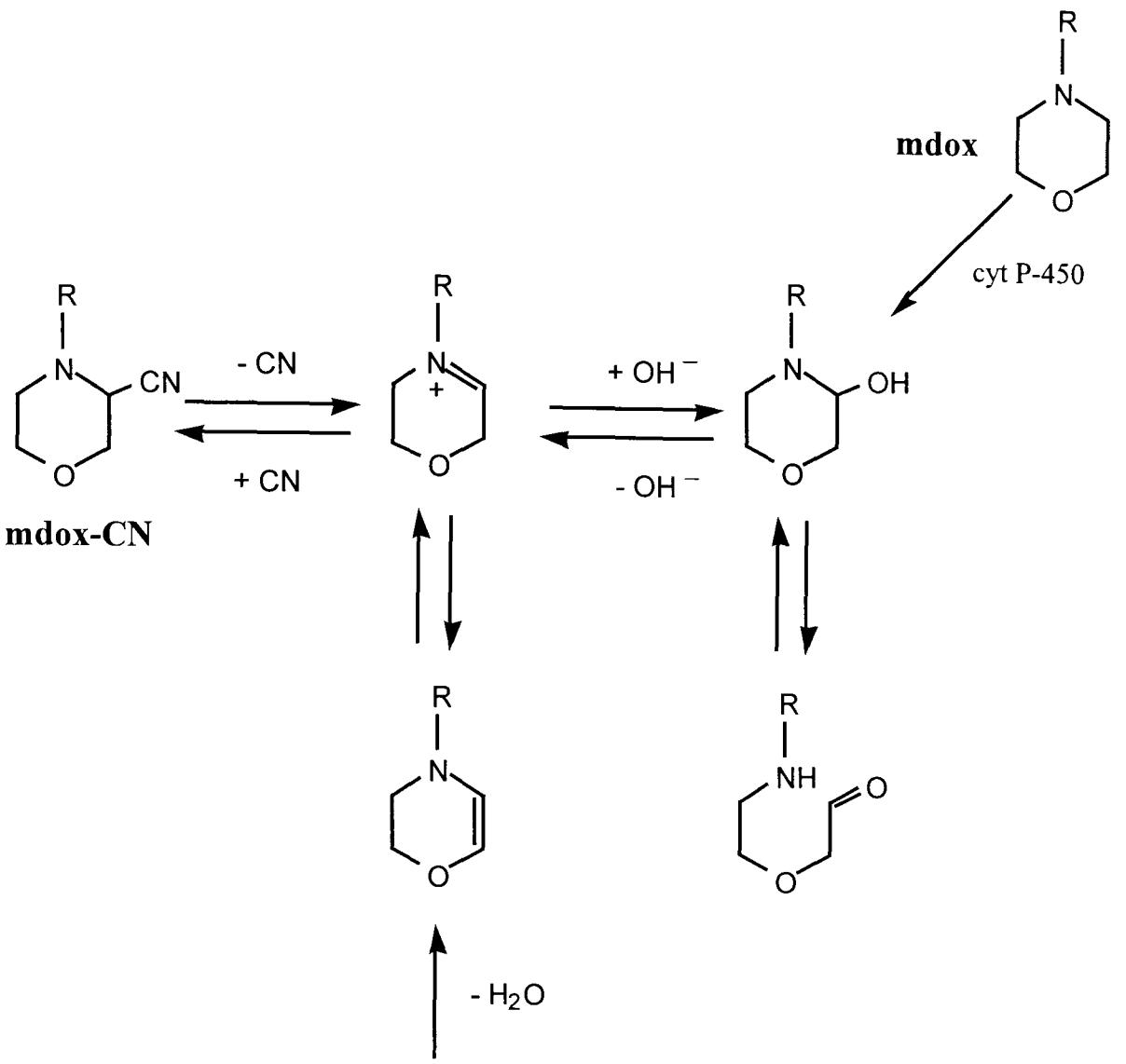


Figure 1.18: Hypothetical chemical and metabolic conversion of morpholino doxorubicin (mdox), cyanomorpholino doxorubicin (mdox-CN) and methoxymorpholino doxorubicin (mmdox) into alkylating species. R denotes the rest of the anthracycline molecule (see figure 1.17).

1.5.3.3 CIRMVENTION OF TOXICITY AND MULTIDRUG RESISTANCE BY MORPHOLINO ANTHRACYCLINES

As mdox and mmdox have intrinsic cytotoxicities comparable to doxorubicin, they can not be considered as true prodrugs. However, preclinical studies have indicated that the enhanced activity of these compounds *in vivo* is not associated with any substantial increase in cardiotoxicity (Sikic *et al*, 1985; Danesi *et al*, 1993). This is probably due to the improvement in therapeutic index of the morpholino compounds upon biotransformation. In addition, the morpholino anthracyclines *per se* are highly potent against doxorubicin-resistant tumour cells. For example, mmdox shows equal activity against the human uterine sarcoma cell line, MES-SA and its doxorubicin-resistant P-glycoprotein positive subline, Dx5 (Lau *et al*, 1994a). Mdox and mmdox both maintain cytotoxicity against the P-gp expressing leukaemia cell line P388/Dx (Streeter *et al*, 1986; Ripamonti *et al*, 1992). The ability of morpholino derivatives to overcome multidrug resistance is thought to relate to their increased lipophilicity, leading to enhanced intracellular uptake and retention (Coley *et al*, 1993; Lau *et al*, 1994b). Available data also suggest that the presence of the morpholino group lowers the affinity of these compounds for the P-glycoprotein pump (Lau *et al*, 1992). Moreover, as they exert their cytotoxic effect through a mode of action primarily involving topoisomerase I rather than topoisomerase II inhibition (Wasserman *et al*, 1990; Duran *et al*, 1996), morpholino anthracyclines also lack cross-resistance against tumour cells presenting the at-MDR phenotype, including the CEM human leukaemia sub-line CEM/VM-1 (Capranico *et al*, 1994, Mariani *et al*, 1994). Studies of the relationship between different chemical modifications on morpholino anthracyclines and their ability to overcome multidrug resistance have demonstrated that while the derivatives containing a morpholino ring at the C-4' position are cytotoxic *in vitro*, unlike the C-3' derivatives, they are completely inactive against disseminated P388 leukaemia (Suarato *et al*, 1995; Ripamonti *et al*, 1996). Thus it can be concluded that positioning of the 4-morpholino ring at the C-3' position of the daunosamine sugar is

essential for *in vivo* activity.

A recent phase II study of the activity of mmdox in non-small-cell lung cancer. renal cancer and other solid tumours showed the main side effects at clinically active doses were myelosuppression, nausea and vomiting, rather than cardiotoxicity (Bakker *et al*, 1998). Similar results were obtained in a previous phase I study (Vasey *et al*, 1995). These results are encouraging, although long-term monitoring is required, bearing in mind the occurrence of late onset of anthracycline-induced cardiotoxicity observed in some patients (see section 1.4.1.2). On a less positive note, the response rates in patients with tumours with intrinsic resistance to chemotherapy were disappointingly low (Bakker *et al*, 1998). Further studies will be required before it can be established whether this is a widespread problem in the clinical use of morpholino anthracyclines.

1.5.4. BIOREDUCTIVE AGENTS AS ANTICANCER PRODRUGS

1.5.4.1 HYPOXIA IN SOLID TUMOURS

One of the predominant features of solid tumours is their highly irregular and disorganized vascularisation, which is usually inadequate for the oxygen requirements of tumour mass. The result is a tendency for cells that are further than 120-150μm from a blood supply to suffer chronic (diffussion limited) hypoxia, which may persist over a period of hours to days (Thomlinson & Gray, 1955). In addition, due to the intermittent opening and closing of blood vessels, tumour cells also experience periods of acute (perfusion-limited) hypoxia, which occurs for up to a few minutes (Brown, 1979). Hypoxia has been demonstrated in a wide variety of human tumours, which may contain areas where oxygen partial pressure is less than 2.5mm Hg, well below the level in the corresponding normal tissues (Nordsmark *et al*, 1994).

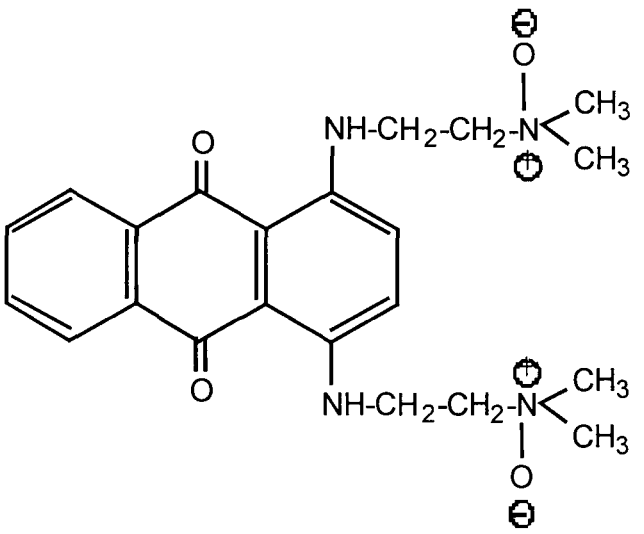
Unfortunately, hypoxia presents a huge problem in the clinical environment, because it almost invariably results in a low response rate to therapy. One reason for this is that certain chemotherapeutic drugs (and radiation) require oxygen to be

maximally cytotoxic (Teicher *et al*, 1981, 1990). Another reason is that most anticancer agents are effective against rapidly dividing cells, but lower oxygen delivery means that the total number of proliferating cells and/or the overall rate of cell proliferation in the tumour is reduced (Tannock, 1968; Bedford & Mitchell, 1974). One way to tackle the problem of hypoxia is by reoxygenation of the tumour between treatments (see Coleman, 1988) or by using radiosensitizers such as the nitroimidazoles, which sensitize hypoxic cells to the effects of radiation by mimicking the electron acceptor properties of oxygen (Jenkins, 1989). However, this technique has limited success because radiosensitization is only temporary. The opposing approach is to exploit hypoxia by developing compounds which are only activated under hypoxic conditions, known as bioreductive agents (reviewed by Brown & Siim, 1996). As these agents kill drug-resistant cells, the combination of a conventional agent and a hypoxia-selective cytotoxin can achieve much greater levels of cell kill than two agents which act on the same cell population. Moreover, drug action is tumour cell specific. Conventional bioreductive agents contain nitro-aromatic or quinone moieties, and can be activated to yield species capable of DNA interaction and/or alkylation (see section 1.5.2.2).

Compounds of this type include the antitumour antibiotic mitomycin C, used in clinical cancer therapy since the 1960s (see Tomasz & Palom, 1997) and the indoloquinone EO9, which has undergone clinical trials (Schellens *et al*, 1994), but with disappointing results. The N-oxides, notably the benztriazine di-N-oxide tirapazamine (Brown, 1993) and the anthraquinone AQ4N, represent a newer class of agents, which will hopefully prove more successful.

1.5.4.2 AQ4N: A HYPOXIA-SELECTIVE CYTOTOXIN

AQ4N, the di-N-oxide derivative of the 1,4-bis-substituted anthraquinone AQ4, is among the most promising new bioreductive agents synthesized to date. The structure of the N-oxide differs from that of its parent compound by the addition of an oxygen atom to the terminal nitrogen of each of the alkylamino side chains. This effectively renders the side chains electrically neutral due to dative covalency between oxygen and the nitrogen lone pair (figure 1.19). The influence of the N-oxide functionality on DNA binding and hence the mechanism of action of AQ4N is discussed later (see section 2.4.2).



AQ4N

Figure 1.19: AQ4N, showing the electrically neutral N-oxide functionality.

In vitro, under anaerobic conditions, AQ4N undergoes cytochrome P-450-dependent bioreductive activation (Raleigh *et al*, 1996; figure 1.20). This results in an approximate 1000-fold increase in potency in human and rodent cell lines compared with the N-oxide (Patterson *et al*, 1994; Wilson *et al*, 1996). Conversely, under aerobic conditions, AQ4N is virtually non-cytotoxic. Preliminary evidence indicates that the CYP3A is the major subfamily responsible for AQ4N metabolism (Patterson & Raleigh, 1998).

Preclinical studies have demonstrated that AQ4N produces an additive effect in

combination with radiotherapy, even when administered up to four days before radiotherapy is initiated (McKeown *et al*, 1995, 1996). Under these conditions, the systemic toxicity is considerably less than that obtained with the other bioreductive agents used (McKeown *et al*, 1995), or is absent altogether (McKeown *et al*, 1996). The effectiveness of AQ4N in the treatment of human malignancies is soon to be determined by way of a phase I clinical trial in the treatment of non-small cell lung cancer (Professor Laurence Patterson, personal communication).

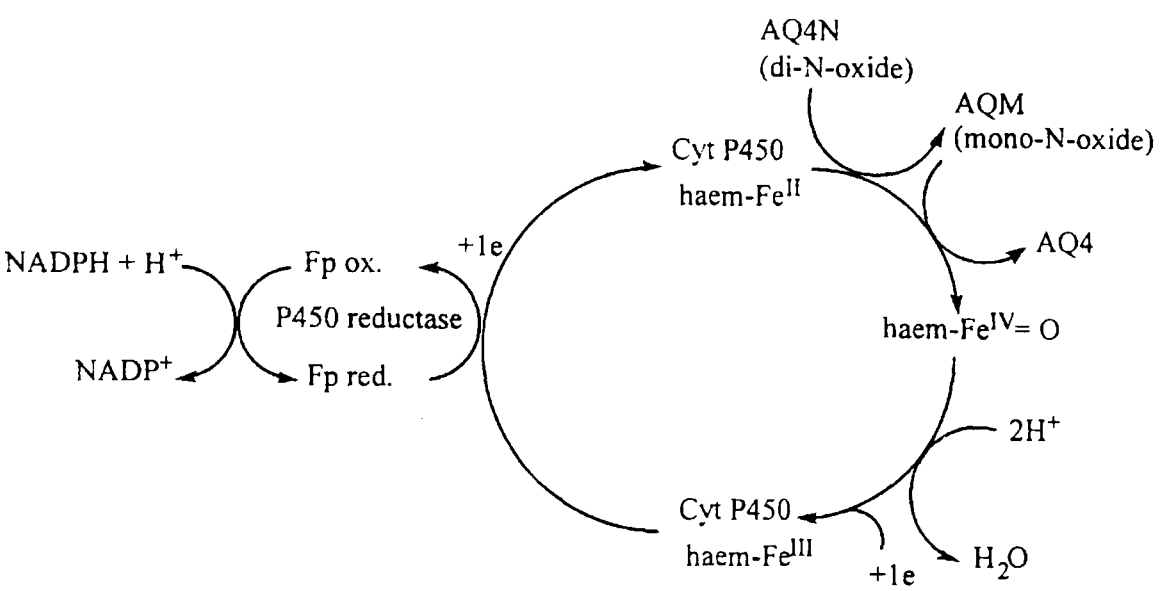


Figure 1.20: Proposed route of bioreduction of AQ4N by cytochrome P-450 (reproduced from Patterson & Raleigh, 1998). Fp ox and Fp red denote the oxidised and reduced forms of the NADPH-cytochrome P-450 reductase flavoprotein.

Hypoxia is only one of many properties unique to tumour tissue (see King, 1996). For example, as discussed earlier (in section 1.5.1), expression of cytochrome P-450 and other drug-metabolizing enzymes may be altered as a consequence of malignancy. This presents considerable scope for the development of agents which are selectively activated in tumour cells or deactivated in normal cells. Compounds of this type already in use include the antitumour agent cyclophosphamide, which is believed to be pharmacologically deactivated to its metabolite carboxyphosphamide in normal tissues.

Over the last decade or so, the metabolism of the anthraquinone antitumour agent mitoxantrone has been studied extensively, and several products of this drug have been identified in humans which indicate metabolic activation may be involved in its mechanism of action (see section 1.2.3). Therefore, it may be feasible to synthesize derivatives of mitoxantrone which undergo selective activation in a similar manner to that already demonstrated for compounds such as cyclophosphamide, the morpholino anthracycline analogues and AQ4N.

1.6 AIMS

In summary, it is now well-established that trapping of topoisomerase II-DNA cleavable complexes on cellular DNA is fundamental to the antitumour activity of drugs such as the anthraquinones and the anthracyclines. Moreover, since the longevity of cleavable complex stabilization is probably a determinant of cytotoxicity, the persistence of drug-induced cleavable complexes observed for mitoxantrone and doxorubicin compared with other topoisomerase inhibitors may contribute to their enhanced potency. Even so, in common with the majority of other topoisomerase II inhibitors, drug-mediated stabilization of cleavable complexes by the anthraquinones and anthracyclines is transitory. This inevitably has a bearing on drug efficacy, in that it allows topoisomerase poisons to be readily removed from the intracellular milieu by the multidrug resistance-associated efflux pumps P-gp and MRP.

Investigation of the molecular aspects of topoisomerase II-anthracycline interactions have identified a number of substituents critical for interference with the enzyme. Drug-DNA interactions have also been studied in detail. The main reason for pursuing this area of research is to gain information that can be used to maximize antitumour efficacy, by structural modification of the original drug. One approach to this has been to synthesize agents which interact with their target covalently, or which do so upon activation. This is true of mmdox, which is activated to a metabolite(s), capable of irreversible binding to DNA. Conceivably, then, it may be possible to design other topoisomerase inhibitors which interact irreversibly with DNA and/or topoisomerase II within the cleavable complex only upon activation. The implications of designing such compounds with respect to multidrug resistance are that permanently bound drug would be unavailable for drug efflux, and that lower levels of topoisomerase II should be required to achieve drug efficacy as the cleavable complexes formed would be more lethal.

Bearing the above information in mind, the aims of this study are

1. To investigate the DNA binding affinity of structural analogues of bis-substituted alkylaminoanthraquinones and to compare this with their ability to inhibit topoisomerase II. Particular attention will be focused on comparing drugs containing cationic alkylamino side chains and those with side chains incorporating an N-oxide moiety. This should (a) provide information regarding the structural requirements necessary to maximize drug activity and (b) lead to information on the effect of the N-oxide functionality on DNA binding and topoisomerase II inhibition which can be used to develop other prodrugs.

2. To synthesize intercalating agents designed to bind covalently to topoisomerase II (i.e. irreversible topoisomerase II inhibitors) in the form of prodrugs. These will contain a masked aldehyde function in the form of an acetal moiety, which can be activated to yield the corresponding aldehyde derivative.

3. To determine the chemical and metabolic conditions under which these drugs (acetalanthraquinones) can be activated *in vitro*.

4. To investigate the interactions of the acetalanthraquinones and their activated products with DNA and topoisomerase II and to relate this to their cytotoxicity.

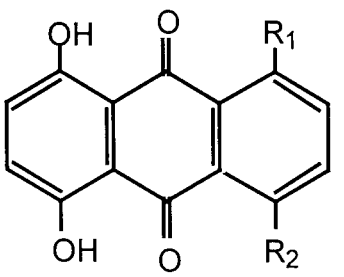
CHAPTER 2: TOPOISOMERASE II INHIBITION AND DNA BINDING OF ALKYLAMINOANTHRAQUINONES

2.1 INTRODUCTION

The purpose of this study was to evaluate the influence of various chromophore and side chain configurations of bis-substituted alkylaminoanthraquinones on DNA binding and topoisomerase II inhibition. The intention was to obtain information on the structural requirements necessary to maximize activity with the aim of developing more potent and selective compounds. In terms of DNA binding, drug structure is thought to influence both the affinity of binding (Kapuscinski *et al*, 1981; Kapuscinski & Darzynkiewicz, 1985; Lown *et al*, 1985) and the kinetic dissociation rate of the DNA-drug intercalatant (Gandecha *et al*, 1985; Krishnamoorthy *et al*, 1986; Denny & Wakelin, 1990). These in turn may influence the longevity of drug-induced cleavable complexes. As the drug side chains may be in close association with topoisomerase II within the drug-trapped cleavable complex (see section 1.3.2.2) particular emphasis was placed on varying the side chain substituents. Since studies have shown that a $HN(CH_2)_2NR'R$ spacer group is essential for cytotoxic activity (Zee-Cheng & Cheng, 1978) most side chains investigated were of this nature (see figure 2.1).

2.2 METHODS AND MATERIALS

V79 (CHL) fibroblasts were derived from Chinese hamster lung (see section 5.2 for details). For preparation of V79 cell nuclear extract, all buffer solutions, centrifuge tubes and utensils used during the extraction were steam sterilized at 110°C prior to use. For the decatenation assay, kinetoplast DNA was supplied by Topogen Inc, Columbus, Ohio, U.S.A. The agarose gel used was low melting. TAE buffer consisted of Tris base, 39.6mM; glacial acetic acid, 18.3mM and EDTA, 1mM. Solutions of BSA (micro grade) were prepared in 100mM Tris buffer, pH 7.5, immediately prior to use.



mitoxantrone R_1=R_2= $NHCH_2CH_2NHCH_2CH_2OH$

AQ3 R_1= $NHCH_2CH_2NHCH_2CH_2OH$, R_2 = $NHCH_2CH_2N(C_2H_5)_2$

AQ4 R_1=R_2= $NHCH_2CH_2N(CH_3)_2$

AQ4N R_1=R_2= $NHCH_2CH_2N(O)(CH_3)_2$

AQ6 R_1= $NHCH_2CH_2NHCH_2CH_2OH$, R_2= $NHCH_2CH_2N(CH_3)_2$

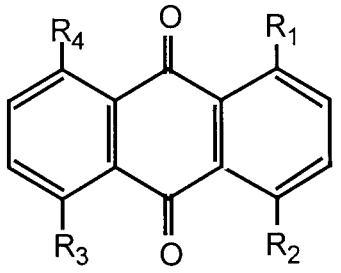
AQ6N R_1= $NHCH_2CH_2NHCH_2CH_2OH$, R_2= $NHCH_2CH_2N(O)(CH_3)_2$

AQ11 R_1=R_2= $NHCH_2CH_2N(CH_2CH_2OH)_2$

AQ13 R_1=R_2= $NHCH_2CH_2CH_2N(C_2H_5)_2$

AQ14 R_1=R_2= $NHCH_2CH_2N(C_2H_5)_2$

AQ20 R_1=R_2= $NHCH_2CH_2N(CH_3)CH_2CH_2OH$



ametantrone R_1=R_2= $NHCH_2CH_2NHCH_2CH_2OH$, R_3=R_4= H

AQ11 R_1=R_2= $NHCH_2CH_2N(CH_2CH_2OH)_2$, R_3=R_4= H

AQ15 R_1=R_3= $NHCH_2CH_2N(C_2H_5)_2$, R_2=R_4= H

AQ16 R_1=R_2= $NHCH_2CH_2N(C_2H_5)_2$, R_3=R_4= H

Figure 2.1: Bis-substituted alkylaminoanthraquinones investigated in this study.

'Mixture A' consisted of Tris-HCl buffer (500mM, pH 7.9) containing dithiothreitol (5mM), triton-X100 (0.05% v/v), $MgCl_2$ (100mM), EDTA (disodium salt; 5mM) and NaCl (final concentration 100mM). Photography of agarose gels was carried out under transillumination at 300nm (UVP Inc.) using a land camera (Kodak, MP-4) loaded with Kodak professional film (Pelica T max 100 4052). For DNA binding studies, all glassware, of 'A' grade specification, was pre-silanized using dichloro-dimethyl silane in 1,1,1-trichloroethane to prevent adsorption of materials to glass surfaces. Excess silanizing solution was removed using methanol (HPLC grade, Fisons, Loughborough, UK). All water was double distilled and stored in glass. The volumes of all solutions were measured using Hamilton glass syringes. The DNA used was calf thymus type 1, sodium salt. The molarity of DNA in solution was determined from the absorbance at 260nm, using a molar extinction coeefficient of 6600 (the value supplied by Sigma Chemical Co.). Isosbestic point determinations were recorded on a Perkin-Elmer Lambda 16 spectrophotometer and spectrophotometric titration on a Beckman DU70 spectrophotometer (single beam). Thermal denaturation studies were recorded on a Perkin-Elmer 552 spectrophotometer, fitted with a Peltier cell connected to a Perkin-Elmer temperature programmer. Anthraquinones were synthesised by Dr M. R. Craven at De Montfort University, Leicester, and were 98-99% pure by HPLC. All other reagents were obtained from Sigma Chemical Co., Poole, Dorset, UK and Aldrich Chemical Co., Gillingham, Dorset, UK, unless otherwise stated, and were 98-99% pure. Where abbreviations have been used for reagents, full names can be found in appendix A8.

2.2.1 TOPOISOMERASE II INHIBITION STUDIES

2.2.1.1 ISOLATION OF TOPOISOMERASE II FROM V79 CELL NUCLEI

Nuclear extract was prepared from V79 cells by a method adapted from Drake *et al* (1987). Culture flasks (15 x 150cm^3) of cells in exponential growth were harvested in non-supplemented RPMI 1640 culture medium (see section 5.2 for details) and the cell density of the suspension was determined using a particle counter (see appendix A7.3). The culture medium was then removed by centrifugation at 1000 x g for 5 min (A14 microcentrifuge, Jouan Ltd., Milan, Italy) and the cells washed with 5ml of 0.01M phosphate buffered saline (pH7.4), containing PMSF 1mM, and soybean trypsin inhibitor (1µg/ml). The cells were centrifuged again at 1000 x g for 5 min and resuspended in 4ml of 5mM potassium phosphate buffer, pH 7.0, containing $MgCl_2$ (2mM), EDTA (0.1mM), PMSF (1mM), benzamidine (1mM), soybean trypsin inhibitor (10µg/ml), leupeptin (50µg/ml) and mercaptoethanol (10mM). The suspension was stirred slowly at 4°C for 15 min, and kept on ice for a further 45 min, with homogenization every 10 min. Lysis of the cells was ascertained using the trypan blue dye exclusion assay (see appendix A1 for details). The lysate was centrifuged at 1000 x g for 10 min and the nuclei washed twice with 4ml of 1mM phosphate buffer, pH 6.5, containing $MgCl_2$, (5mM), EGTA (1mM), glycerol (10% w/w), NaCl (100mM), PMSF (1mM), benzamidine (1mM), soybean trypsin inhibitor (10µg/ml), leupeptin (50µg/ml) and mercaptoethanol (10mM). The washed nuclei were then resuspended in 2ml of 5mM phosphate buffer, pH 7.0, containing $MgCl_2$ (2mM), EDTA (0.1mM), glycerol (10% w/w), PMSF (1mM), benzamidine (1mM), soybean trypsin inhibitor (10µg/ml), leupeptin (50µg/ml) and mercaptoethanol (10mM). The fraction containing topoisomerase II was extracted from the suspension by stirring with NaCl, at a final concentration of 0.35M, for 60 min. The extract was centrifuged at 2250 x g for 15 min, then at 20 000 x g for 40 min, and the supernatant stored in sterile vials at -70°C. The procedure followed for determining the protein content of the V79 cell nuclear extract was as described in appendix A2.

2.2.1.2 OPTIMIZATION OF THE CONCENTRATION OF TOPOISOMERASE II REQUIRED FOR DECATENATION OF KDNA

The decatenation of kinetoplast DNA (kDNA; from *Crithidia fasciculata*) was used to assay the level of topoisomerase II activity of the V79 cell nuclear extract. Dilutions of nuclear extract (1 in 2 to 1 in 10) were prepared in Tris buffer (100mM, pH 7.5). The assay components were carefully pipetted onto the side of Eppendorf tubes in the order: 2.15µl kDNA (93.15µg/ml), 2.0µl 'Mixture A', 2.0µl ATP (12.5mM; in sterile distilled water), 3.0µl BSA (200µg/ml; in sterile distilled water), 2.0µl nuclear extract at various dilutions and sterile distilled water to 20µl.

The reaction was initiated by microcentrifugation at 14 000 x g for 10 sec, followed by incubation at 37°C for 30 min. The reaction was stopped by the addition of 2.0µl of a solution containing SDS (10% w/v) bromophenol blue (0.25% w/v) and sucrose (1.2M) to the side of the tube, and centrifuging as described above. The samples (20.0µl) were loaded onto a 1% agarose gel submerged in TAE buffer containing ethidium bromide (0.5µg/ml), and electrophoresed for 4 h at 37V/2mA. The gel was destained in TAE buffer for 1 h, and then photographed under UV transillumination for 30 sec. The negative was developed in the dark in ID11 developer (Ilford) for 1 min and fixed for 30 min (using Hypam fixer, Ilford)

2.2.1.3 OPTIMIZATION OF THE INCUBATION TIME FOR DECATENATION OF KDNA

All samples were incubated at 37°C for various time periods between 0 and 60 min. Each incubate contained 2.15µl kDNA (93.15µg/ml), 2.0µl 'Mixture A', 2.0µl ATP (12.5mM; in sterile distilled water), 3.0µl BSA (200µg/ml; in sterile distilled water), 2.0µl V79 nuclear extract (1 in 2 dilution) and sterile distilled water to 20µl. The procedure thereafter was as described in section 2.2.1.2.

2.2.1.4 OPTIMIZATION OF ATP AND MAGNESIUM ION LEVELS FOR DECATENATION OF KDNA

Samples were incubated at 37°C in the presence and absence of ATP and Mg^{2+} for 0 to 60 min. Each incubate consisted of 2.15µl kDNA (93.15µg/ml), 2.0µl 'Mixture A' with or without $MgCl_2$ (10.00-200mM), 2.0µl ATP (0-15mM), 3.0µl BSA (200µg/ml; in sterile distilled water), 2.0µl V79 nuclear extract (1 in 2 dilution) and sterile distilled water to 20µl. The procedure thereafter was as described in section 2.2.1.2.

2.2.1.5 THE EFFECT OF ALKYLAMINOANTHRAQUINONES ON THE DECATENATING ACTIVITY OF TOPOISOMERASE II

Samples were incubated at 37°C for 30 min with various concentrations of drug, ranging from 1nM to 100µM. Each incubate consisted of kDNA (93.15µg/ml), 2.0µl 'Mixture A' 2.0µl ATP (12.5mM; in sterile distilled water), 3.0µl BSA (200µg/ml; in sterile distilled water), 2.0µl of sterile filtered drug (in distilled water), 2.0µl V79 nuclear extract (1 in 2 dilution) and sterile distilled water to 20µl. Nuclear extract and/or drug were omitted from the control incubations, and replaced by 'extraction buffer' (see section 2.2.1.1) and/or sterile distilled water respectively. The procedure thereafter was as described in section 2.2.1.2.

2.2.2 DNA BINDING STUDIES

2.2.2.1 THE EFFECT OF CALF THYMUS DNA ON THE SPECTRAL PROPERTIES OF ALKYLAMINOANTHRAQUINONES

Solutions of drug were prepared in Tris buffer (Tris base, 8mM and NaCl, 0.05M, pH 7.2, in double distilled water) and added to silanized 'A' grade volumetric flasks (10ml) to give a final drug concentration of 1.5×10^{-5}M. Calf thymus DNA (in the same buffer) at a concentration of approximately 1.5×10^{-3}M was added to each drug solution to give DNA to drug ratios of 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 15.0 to 1.0. The absorbance spectra of the solutions were then recorded between 400 and 700nm against

a Tris buffer blank, and superimposed. The procedure was repeated using DNA and drug solutions prepared in buffer containing Tris base, 8mM and NaCl, 0.5M, pH 7.2.

2.2.2.2 SPECTROPHOTOMETRIC TITRATION OF ALKYLAMINO-ANTHRAQUINONES AGAINST CALF THYMUS DNA

A volume of precisely 3.0ml of drug solution at a concentration of 2.5×10^{-5}M in Tris buffer (Tris base 8mM and NaCl 0.5M, pH 7.2, in double distilled water) was added to three matched quartz cuvettes of 1cm pathlength. Tris buffer (3ml) was used as a blank to calibrate the spectrophotometer. Volumes of 12 x 10, 6 x 20, 8 x 40 and 5 x 100μl of calf thymus DNA (concentration 1.25×10^{-3}M) in Tris buffer, were added sequentially to each of the drug solutions with a Hamilton syringe. Each solution was then mixed gently for 15 sec and left for 5 min to allow the DNA/drug mixture to equilibrate. After equilibration, the absorbance of each sample was measured at the λmax of the unbound drug. Scatchard plot analysis was use to determine the values of K (the affinity constant of drug for DNA) and n (the number of drug binding sites per DNA phosphate residue).

2.2.2.3 THE EFFECT OF ALKYLAMINOANTHRAQUINONES ON THE THERMAL DENATURATION OF CALF THYMUS DNA

A solution of drug (3.0ml) in Tris buffer (Tris base, 8mM and NaCl, 0.05M, pH 7.2, in double distilled water) was added to approximately 6ml of double distilled water, and de-gassed by sonication for 15 min. A solution of calf thymus DNA in the same Tris buffer (600μl) at a concentration of 1.5×10^{-3}M was then added and the mixture diluted to 10.0ml with double distilled water, to give a DNA to drug ratio of 10:1. Exactly 3.0ml of this solution was transferred to a quartz cuvette (1cm pathlength) which was then tightly stoppered and left to equilibrate at 60°C for 15 min. The absorbance was measured at 260nm against a non-heated Tris buffer blank, and further measurements were taken at 1°C intervals (0.5°C/min) up to 110°C or until there was no

further change in absorbance. In determining the melting temperature of DNA alone, drug solution was replaced by an equal volume of Tris buffer.

2.3 RESULTS

2.3.1 TOPOISOMERASE II INHIBITION STUDIES

2.3.1.1 THE DECATENATION ASSAY

The decatenation assay is based on the ability of type II topoisomerases to resolve high molecular-weight catenated DNA networks to individual DNA circles and intermediate-sized catenated complexes, which move rapidly into the gel upon electrophoresis (Miller *et al*, 1981). In the presence of topoisomerase II poisons, decatenation is inhibited, and the intact networks remain in the loading well. The catenated DNA substrate used in this study was kinetoplast DNA extracted from the mitochondria of the trypanosome *Crithidia fasciculata*, which consists of 2.5 kb minicircles (93-95%) and 40kb maxicircles (Marini *et al*, 1980).

A 2.0µl volume of a 1 in 2 dilution of V79 cell nuclear extract, corresponding to 855ng of protein per incubate (see appendix A2), achieved complete decatenation of 200ng kDNA in 30 min at 37°C (equivalent to 7.8 µg DNA/min/mg protein). Decatenation was also found to be ATP- and Mg^{2+}- dependent, the optima being 12.5mM and 100mM respectively. These conditions were maintained for the subsequent screening of drugs.

2.3.1.2 THE EFFECT OF ALKYLAMINOANTHRAQUINONES ON TOPOISOMERASE II ACTIVITY

The effect of the alkylaminoanthraquinones on the decatenation of kDNA is shown in table 2.1. It can be seen that mitoxantrone completely inhibited decatenation at a concentration of 0.75µM, indicated by intact networks remaining in the loading wells (figure 2.2). The mitoxantrone analogues AQ4 and AQ6 inhibited decatenating activity at 1.5µM and 1.0µM respectively, both within the same range as mitoxantrone (figures

2.3 and 2.4). In contrast, total inhibition of decatenation by the N-oxides AQ4N and AQ6N required concentrations of 50μM and 10μM respectively, 33 times and 10 times that required for their respective parent compounds (figures 2.3 and 2.4). Control incubations (without drug) did not inhibit decatenation of kDNA, as indicated by migration of decatenated circles into the gel.

Table 2.1: Inhibition of topoisomerase II-mediated decatenation of kDNA by bis-substituted alkylaminoanthraquinones

DRUG	**alkylamino side chains** [a]	**inhibitory drug concentration (μM)** [b]
mitoxantrone	R_1 = $-NHCH_2CH_2OH$ R_2 = $-NHCH_2CH_2OH$	0.75
AQ4	R_1 = $-N(CH_3)_2$ R_2 = $-N(CH_3)_2$	1.5
AQ4N	R_1 = $-NO(CH_3)_2$ R_2 = $-NO(CH_3)_2$	50.0
AQ6	R_1 = $-NHCH_2CH_2OH$ R_2 = $-N(CH_3)_2$	1.0
AQ6N	R_1 = $-NH\ CH_2CH_2OH$ R_2 = $-NO(CH_3)_2$	10.0

a: structure of alkylamino side chains terminal to the aliphatic nitrogen at positions 1 and 4 of the anthraquinone chromophore (see figure 2.1); b: drug concentration causing total inhibition of kDNA decatenation.

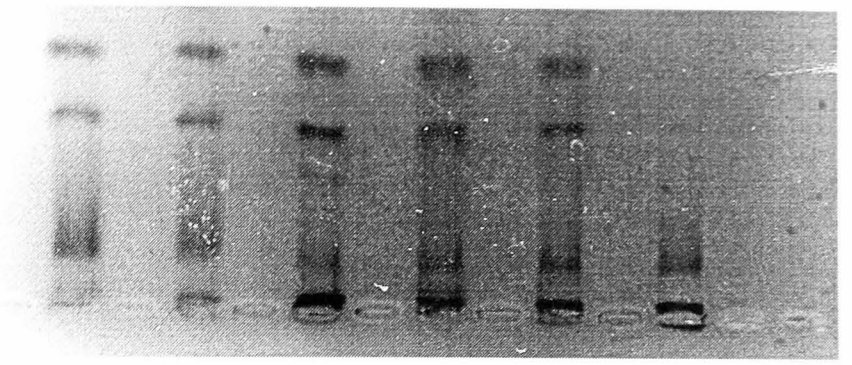


Figure 2.2: Inhibition of topoisomerase II-mediated decatenation of kDNA by mitoxantrone. Lane 1 (left): kDNA + topo II; lanes 2-6: kDNA, topo II + 0.05, 0.1, 0.2, 0.5 and 0.75μM mitoxantrone respectively.

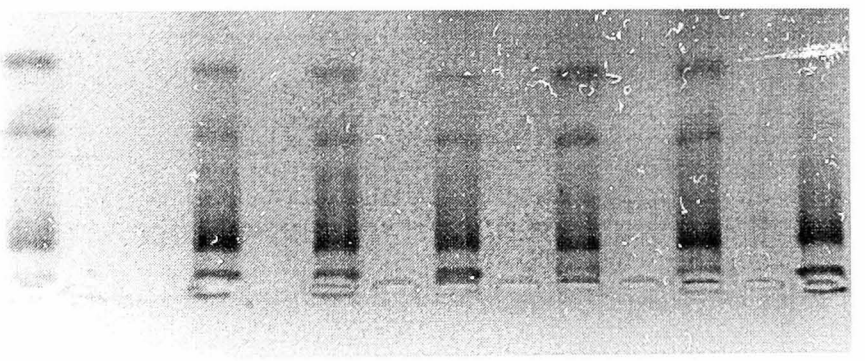


Figure 2.3: Inhibition of topoisomerase II-mediated decatenation of kDNA by AQ4 and AQ4N. Lane 1 (left): kDNA + topo II; lanes 2-4: kDNA, topo II + 0.5, 0.75 and 1.0μM AQ4 respectively. Lanes 5-7: kDNA, topo II + 10, 25 and 50μM AQ4N respectively.

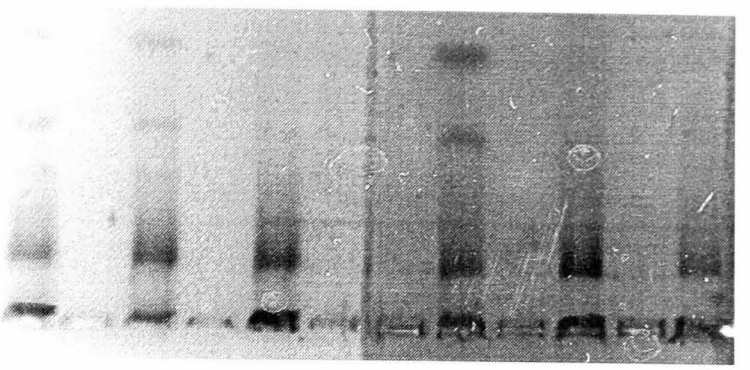


Figure 2.4: Inhibition of topoisomerase II-mediated decatenation of kDNA by AQ6 and AQ6N. From left: lanes 1-3: kDNA, topo II + 0.5, 0.75 and 1.0μM AQ6 respectively. lanes 4-6: kDNA, topo II + 5, 10 and 25μM AQ6N respectively.

2.3.2 DNA BINDING STUDIES

2.3.2.1 DNA-DRUG INTERACTIONS

Due to their amphipathic nature, alkylaminoanthraquinones can participate in two different types of DNA binding. Firstly, their planar tricyclic chromophore allows them to intercalate between the DNA base pairs (Double & Brown, 1975). This process is stabilized by hydrophobic interactions between the base pairs and the π orbitals of the drug chromophore. Intercalation induces several changes in the properties of alkylaminoanthraquinones, including changes in their absorption spectra, and also stabilizes the DNA helix to thermal denaturation. Secondly, the cationic (amino) functions of the drug side chains can interact with DNA via the negatively charged DNA phosphate groups, which further stabilizes the DNA-drug complex (Kapuscinski *et al*, 1981; Foye *et al*, 1982; Lown *et al*, 1984). As this type of binding occurs on the exterior of the DNA helix, it is much weaker than intercalation, and is subject to the influence of the ionic concentration of the solution (Jones *et al*, 1980). The significance of this will become apparent later.

2.3.2.2 SPECTRAL PROPERTIES OF ALKYLAMINO-ANTHRAQUINONES

Due to the donation of electrons by the aromatic nitrogen atoms into their π conjugated ring system, anthraquinone-based compounds have a characteristic absorbance in the visible region of the spectrum. However, on binding to DNA, these UV and visible properties are altered (Waring, 1981). Upon intercalation, a bathochromic shift (a shift to a longer wavelength) usually occurs, and the molar extinction at the λmax decreases, producing a hypochromic shift. These shifts vary according to the ratio of DNA:drug present. In addition, the drug may display an isosbestic point. This is obtained by varying the concentration of DNA that is added to a fixed concentration of drug and superimposing the spectra recorded between two wavelengths. The presence of an isosbestic point indicates that one type of binding complex is formed between the drug and DNA (Waring, 1981).

Table 2.2 shows the effect of calf thymus DNA on the spectral properties of the alkylaminoanthraquinones. Figure 2.5 shows the spectrum for AQ15 at DNA to drug ratios between 0:1 and 15:1 in the visible region of the spectrum, using buffer containing 0.05M NaCl. Figure 2.6 shows the spectrum for AQ15 at these DNA:drug ratios at an NaCl concentration of 0.5M NaCl. The spectra for AQ16 at ionic concentrations 0.05M and 0.5M are shown in figures 2.7 and 2.8 respectively. The red shift of the maxima and the hypochromicity are consistent with intercalative binding, and have previously been observed for mitoxantrone and ametantrone in the presence of double stranded polymers (Kapuscinski *et al*, 1981; Kapuscinski & Darzynkiewicz, 1985; Lown *et al,* 1985). All parent compounds in the anthraquinone series showed hypochromic and bathochromic shifts in the presence of calf thymus DNA and were fully bound to DNA at a DNA:drug ratio of 15:1.

Table 2.2: Spectral properties of bis-substituted alkylaminoanthraquinones in the presence of calf thymus DNA

DRUG	isosbestic point (nm)	shift in λmax (nm) [a]	% decrease in extinction [b]
mitoxantrone	621	17	15
ametantrone	636	12	16
AQ3	623 / 667	18	15
AQ4	622	17	15
AQ4N	---	---	---
AQ6	620	17	12
AQ6N	621	7	9
AQ11	659*	14	19
AQ12	642	2	8
AQ13	620 / 660	19	11
AQ14	667	18	16
AQ15	---	4	16
AQ16	639	4	15
AQ20	637	17	20

a: shift in λmax at a DNA to drug ratio of 15:1; b: difference between λmax of free drug and fully bound drug (hypochromic shift). * indicates an isosbestic point was observed only at higher DNA:drug ratios. All experiments were performed in 0.5M NaCl/ 0.008M Tris buffer.

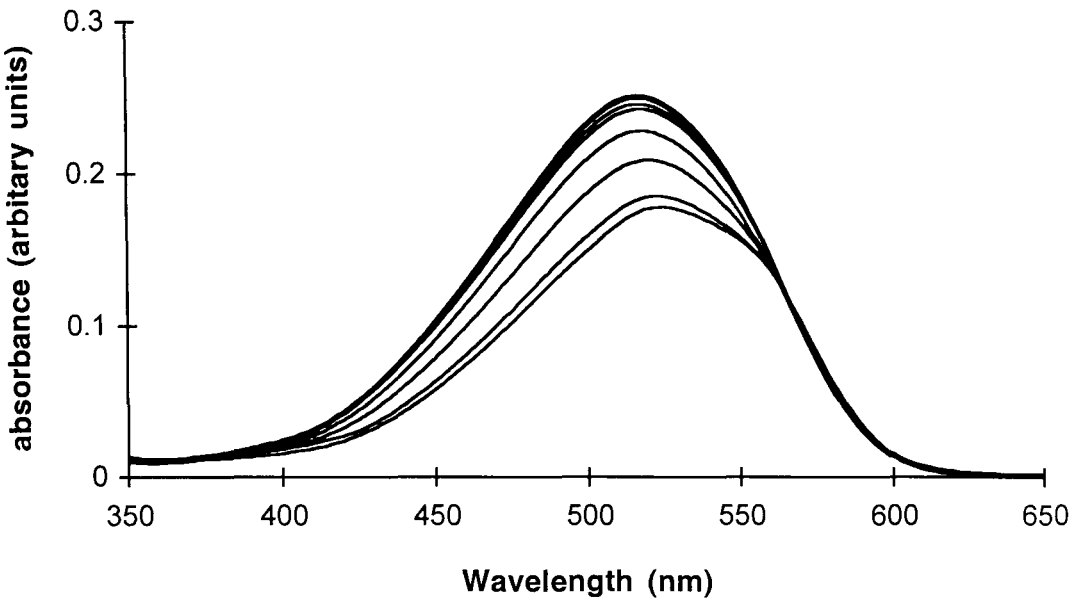


Figure 2.5: Spectral properties of AQ15 in the presence of calf thymus DNA and 0.008M Tris/0.05M NaCl.

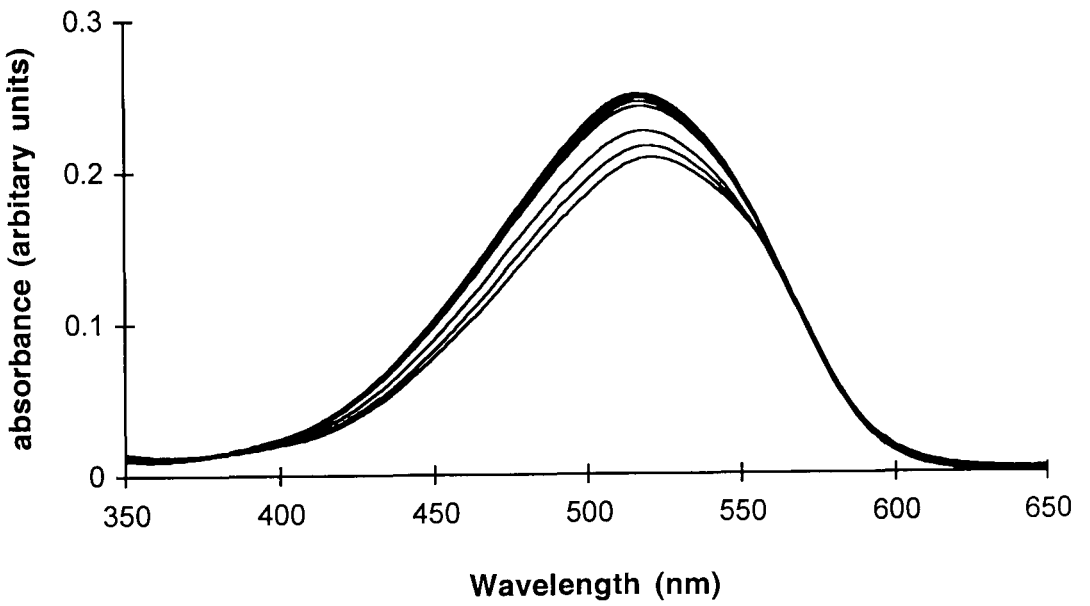


Figure 2.6: Spectral properties of AQ15 in the presence of calf thymus DNA and 0.008M Tris/0.5M NaCl.

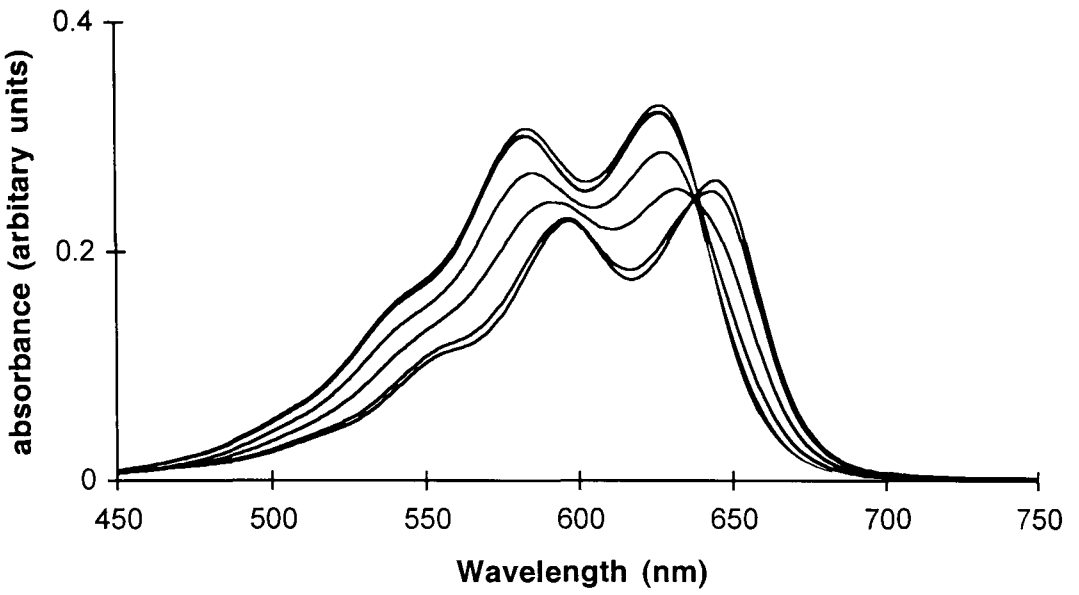


Figure 2.7: Spectral properties of AQ16 in the presence of calf thymus DNA and 0.008M Tris/0.05M NaCl.

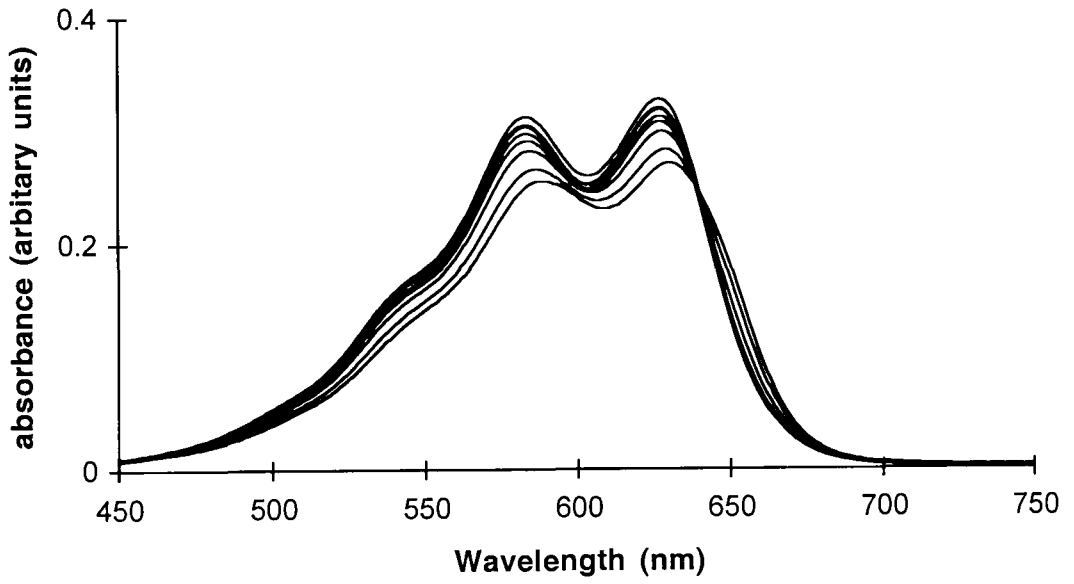


Figure 2.8: Spectral properties of AQ16 in the presence of calf thymus DNA and 0.008M Tris/0.5M NaCl.

Apart from AQ11 and AQ15, all parent compounds gave isosbestic points at a concentration of 0.5M NaCl, consistent with a single-type of DNA-drug binding complex. However, for a number of drugs, using 0.05M NaCl, the isosbestic point was lost, particularly at lower DNA:drug ratios, indicating the presence of more than one form of DNA-drug complex (data not shown). This effect was observed only for compounds that were dihydroxylated at the 5 and 8 positions of the chromophore and was more evident for the compounds containing side chain hydroxy groups.

Compounds hydroxylated at the 5 and 8 positions of the chromophore generally showed increased bathochromic and hypochromic shifts compared with the non-hydroxylated analogues. The spectrum of the di-N-oxide, AQ4N, showed little change as the DNA concentration was increased, indicating intercalation did not take place, while the mono-N-oxide, AQ6N, showed moderate bathochromic and hypochromic shifts, and isosbestic points at both 0.5M and 0.05M NaCl. Although the spectra for AQ15 all converged at ~ 557nm, they did not cross and thus a true isosbestic point was not obtained. For AQ11, the spectra crossed through a single point only at higher DNA:drug ratios.

2.3.2.3 DETERMINATION OF THE AFFINITY OF ALKYLAMINO-ANTHRAQUINONES FOR CALF THYMUS DNA BY SPECTROPHOTOMETRIC TITRATION

On sequential addition of DNA to a solution of DNA intercalating agent, there is a progressive decrease in extinction (hypochromic shift) at the λmax of the unbound drug (as DNA-bound drug has different λmax to free drug-see section 2.3.2.2). When there is no further decrease in extinction, the drug is fully bound to DNA. The fraction of bound drug (α), at any particular DNA concentration can be calculated from the equation

$$\alpha = \frac{Ef - Eobs}{Ef - Eb}$$

where E_f is the extinction of free drug at the λmax, E_{obs} is the extinction of the sample

after each addition of DNA and E_b is the extinction of the bound drug, estimated from a plot of

$$E_{obs} \text{ vs } \frac{[DNA]}{C_T}$$

C_T being the total drug concentration available for binding. This allows the affinity of the drug for DNA to be quantified using the Scatchard plot:

$$r/c = -Kr + Kn$$

where r is the number of moles of ligand bound per nucleotide, equal to αC_T /[DNA], αC_T is the concentration of bound drug, and c is the concentration of free drug, $(1-\alpha)C_T$ (Blake & Peacocke, 1968).

K (the DNA affinity constant) can be calculated by linear regression and n (the number of drug molecules bound per DNA phosphate) from the x-axis intercept. For this method to be valid, the drug must obey Beer-Lambert's Law within the range of drug concentrations used, so that the fraction of bound drug is proportional to the fractional decrease in extinction. There must also be a clear isosbestic point, as this indicates that there is only one type of spectroscopically distinct DNA-drug complex present (see section 2.3.2.2).

Results for K and n are shown in table 2.3. Individual absorbance values deviated no more than ± 5% from the mean. The Scatchard plots for mitoxantrone, ametantrone, AQ6 and AQ6N are shown in figures 2.9 to 2.12. All drugs were found to obey Beer-Lambert's Law up to a concentration of 1 x 10^{-4}M in 0.5M NaCl, pH 7.2 (results not shown). As in several cases, isosbestic points could be obtained using 0.5M but not 0.05M NaCl, spectrophotometric titration was performed using 0.5M NaCl for all compounds. At this ionic concentration, it was anticipated that surface binding would be minimal, so only one K value was obtained, corresponding to intercalative binding.

As insignificant hypochromic and bathochromic shifts were obtained for AQ4N, and no isosbestic point was obtained for AQ11 or AQ15, K and n values for these compounds were not determined.

Table 2.3: Values of K and n for bis-substituted alkylaminoanthraquinones obtained by spectrophotometric titration with calf thymus DNA

DRUG	K ($x10^6 M^{-1}$)	n
mitoxantrone	4.2	0.12
ametantrone	3.3	0.12
AQ3	4.6	0.13
AQ4	5.0	0.13
AQ4N	nd	nd
AQ6	4.6	0.13
AQ6N	3.6	0.13
AQ11	nd	nd
AQ12	1.6	0.12
AQ13	3.9	0.13
AQ14	4.6	0.13
AQ15	nd	nd
AQ16	2.2	0.13
AQ20	4.7	0.14

K represents the affinity constant and n the number of drug binding sites per DNA phosphate. nd = not determined. Values represent the mean of three separate experiments performed in triplicate. The absorbance values obtained for each experiment deviated no more than ± 5% from the mean.

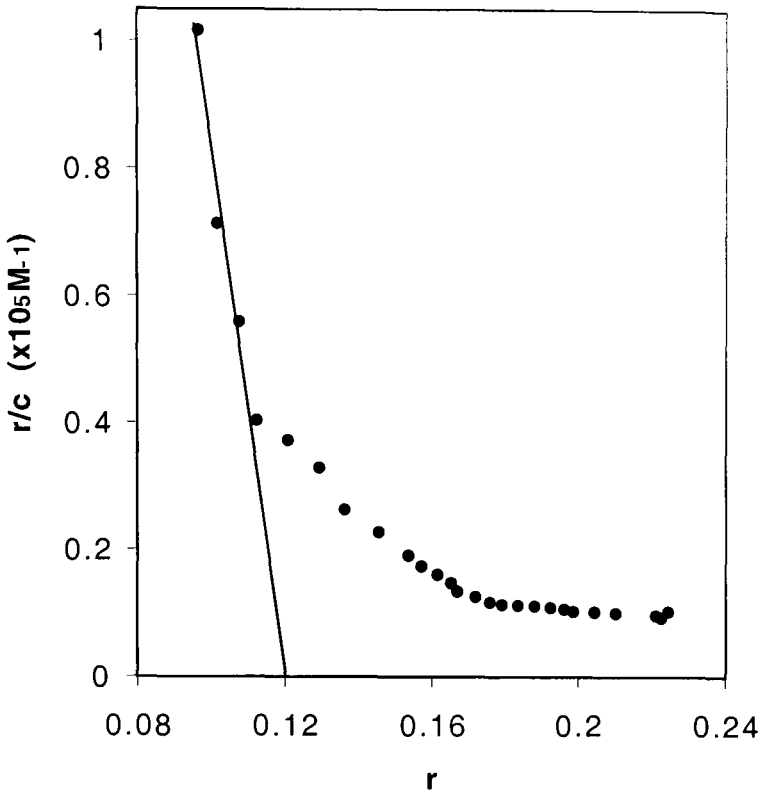


Figure 2.9: Scatchard plot obtained from spectrophotometric titration of mitoxantrone with calf thymus DNA. Values shown represent the mean of three separate experiments.

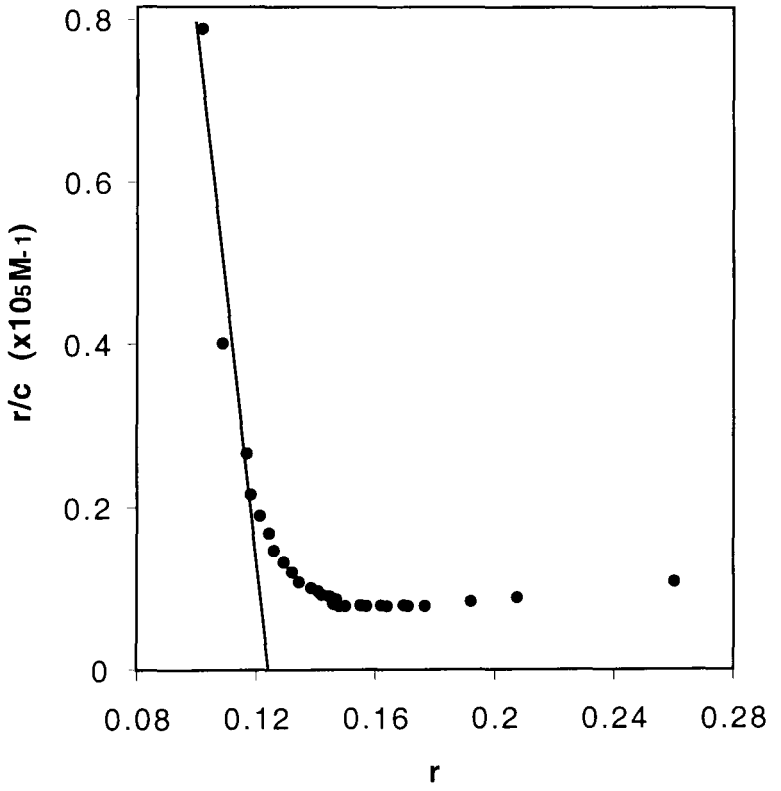


Figure 2.10: Scatchard plot obtained from spectrophotometric titration of ametantrone with calf thymus DNA. Values shown represent the mean of three separate experiments.

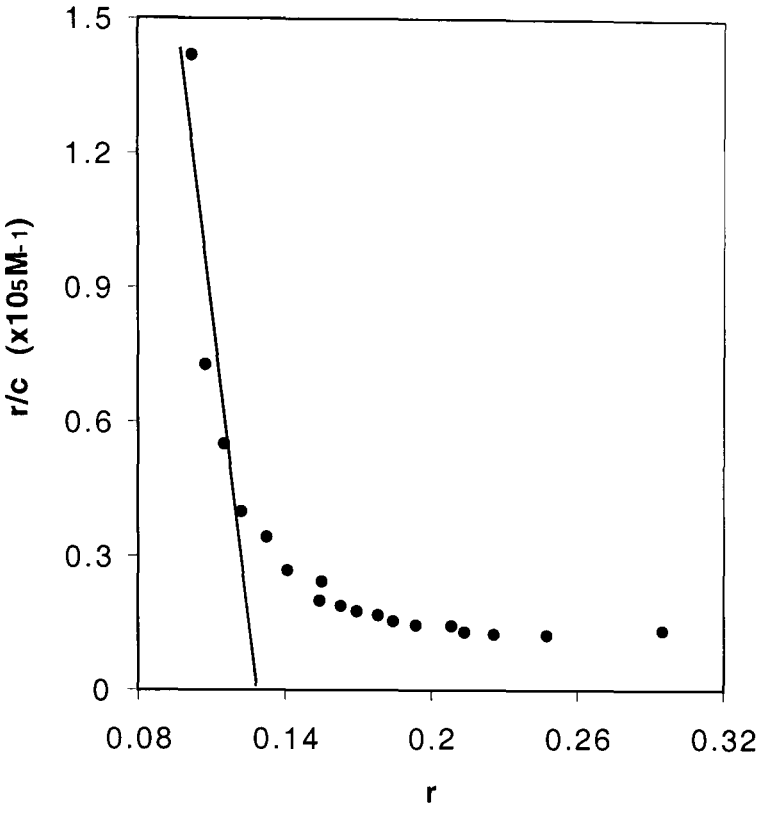


Figure 2.11: Scatchard plot obtained from spectrophotometric titration of AQ6 with calf thymus DNA. Values shown represent the mean of three separate experiments.

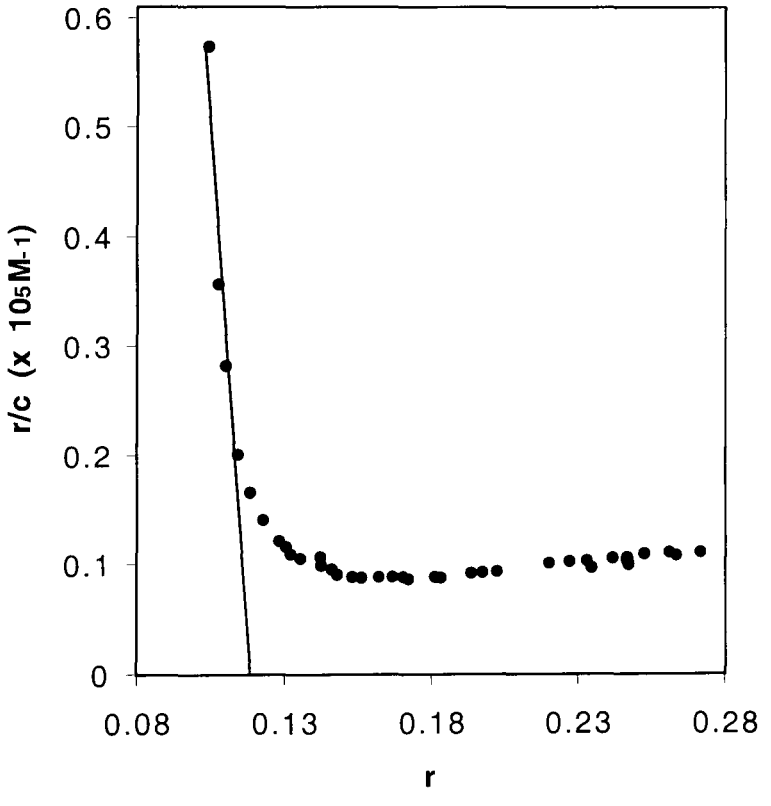


Figure 2.12: Scatchard plot obtained from spectrophotometric titration of AQ6N with calf thymus DNA. Values shown represent the mean of three separate experiments.

The K values for all compounds at 0.5M were of the same order of magnitude (10^6M^{-1} range). However, the 5,8-dihydroxy substituted analogues generally had higher K values than the non-hydroxylated analogues (3.94-4.95 x 10^6M^{-1} compared to 1.63-3.25 x 10^6M^{-1}). Thus, for the parent compounds, hydroxylation of the chromophore had a greater effect on K than variations in the side chain groups, as long as their cationic nature was maintained. However, modification of the terminal nitrogen on the alkylamino side chain to form an N-oxide resulted in a large decrease in DNA binding affinity. AQ6N, in which one cationic alkylamino side chain was retained, gave a moderate K value (3.64 x 10^6M^{-1}) .

2.3.2.4 THERMAL DENATURATION OF CALF THYMUS DNA IN THE PRESENCE OF ALKYLAMINOANTHRAQUINONES

In solution, the two strands of the DNA double helix are essentially held together by hydrogen bonding between complementary bases and by base pair stacking. However, upon heating, the DNA helix unwinds and the two strands separate. This process is known as thermal denaturation, and can be measured by the increase in absorbance of the solution at 260nm (Marmur & Doty, 1959). When drug molecules intercalate between the base pairs of DNA, its structure is stabilized so that more heat energy is required to cause denaturation. In contrast, if the drug binds externally to the helix, there is usually little if any increase in the temperature at which denaturation takes place. Thus, the difference in the temperature at which denaturation takes place for DNA-drug complexes compared to DNA alone (known as ΔTm) gives an indication of the degree of intercalation for a particular compound.

The melting profile for DNA is shown in figure 2.13 and ΔTms for several of the drugs are shown in figures 2.14. to 2.20. Data are summarized in table 2.4. Tm was determined as being the temperature corresponding to the midway point on the slope of increasing absorbance, from a plot of A vs T. First derivative plots, the change in absorbance per degree rise in temperature vs. temperature (dA/dT vs. T), allowed more

subtle changes in the melting process to be seen, particularly biphasic trends, where dA/dT showed a maximum at two temperatures.

The Tm of calf thymus DNA in the absence of drug was 70.6 ± 0.5°C. The parent compounds in the anthraquinone series all caused considerable increases in Tm, the 5,8 dihydroxylated anthraquinones generally showing larger increases than the non-hydoxylated compounds (13.5-28.0 °C compared to 13.9-21.5 °C), The effect of the di-N-oxide AQ4N on melting temperature was minimal (1.8°C), whilst the mono-N-oxide, AQ6N, caused a modest increase (7.0°C). The first derivative plots of AQ4N, AQ11 and AQ12 were symmetrical, as was that for DNA itself. First derivative plots of all other compounds (notably AQ3, AQ4 and AQ13) were found to be of a biphasic nature. Drug solutions heated without DNA showed no spectral changes over a time period of 3 hours.

Table 2.4: ΔTm values of calf thymus DNA in the presence of bis-substituted alkylaminoanthraquinones

DRUG	Tm (°C)	ΔTm [a]	biphasic first derivative plot [b]
mitoxantrone	97.0 ± 0.6	26.4	yes
ametantrone	92.1 ± 0.1	21.5	yes
AQ3	86.8 ± 1.4	16.2	yes
AQ4	88.3 ± 0.5	17.7	yes
AQ4N	72.4 ± 0.8	1.8	no
AQ6	98.6 ± 0.5	28.0	yes
AQ6N	77.6 ± 1.2	7.0	yes
AQ11	84.2 ± 0.1	13.6	no
AQ12	84.5 ± 0.3	13.9	no
AQ13	88.7 ± 0.2	18.1	yes
AQ14	89.2 ± 0.2	18.6	yes
AQ15	89.6 ±0.7	19.0	yes
AQ16	86.4 ± 0.3	15.8	yes
AQ20	93.8 ± 0.8	23.2	yes

a: difference in melting temperature of DNA in the presence and absence of drug (Tm of calf thymus DNA= 70.6 ± 0.5°C); b: first derivative plots (dA/dt vs. T) displayed absorption maxima at two temperatures. Values represent the mean ± sd for three separate experiments.

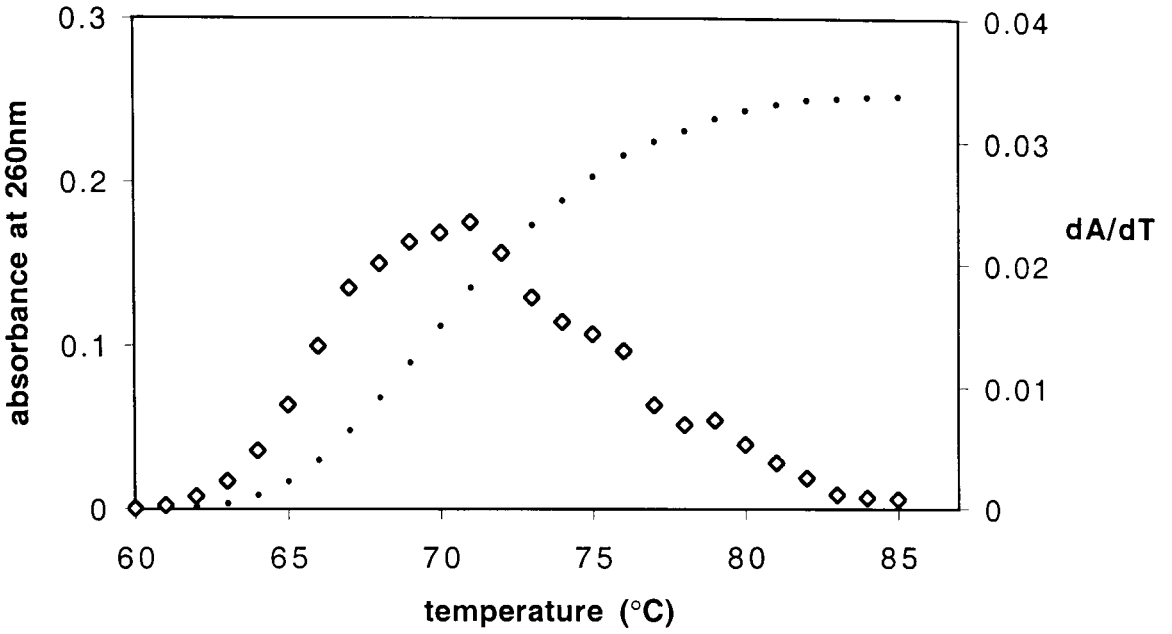


Figure 2.13: Melting profile of calf thymus DNA, showing A vs T (•) and dA/dT vs T (◊). Values shown represent the mean of three separate experiments.

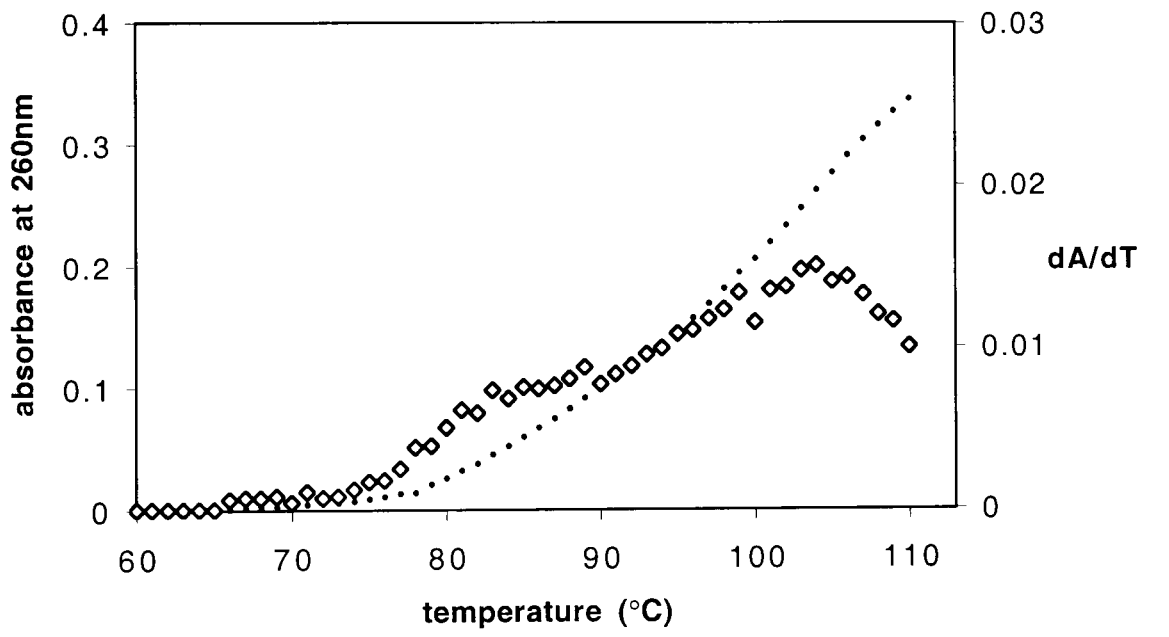


Figure 2.14: Melting profile of calf thymus DNA in the presence of mitoxantrone, showing A vs T (•) and dA/dT vs T (◊). Values shown represent the mean of three separate experiments.

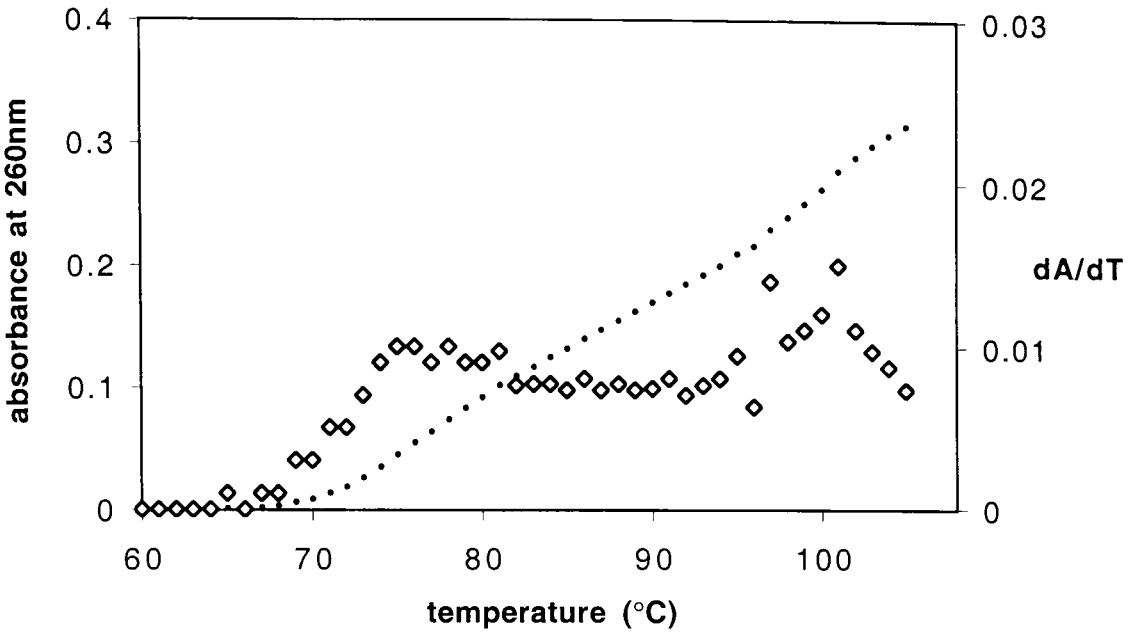


Figure 2.15: Melting profile of calf thymus DNA in the presence of AQ4, showing A vs T (•) and dA/dT vs T (◊). Values shown represent the mean of three separate experiments.

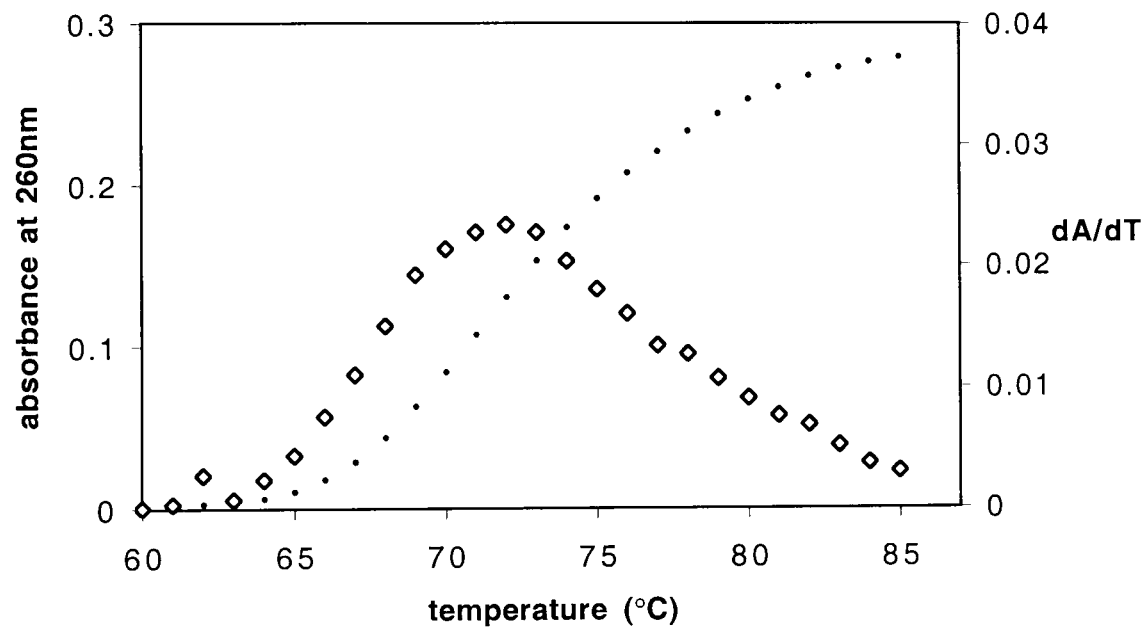


Figure 2.16: Melting profile of calf thymus DNA in the presence of AQ4N, showing A vs T (•) and dA/dT vs T (◊). Values shown represent the mean of three separate experiments.

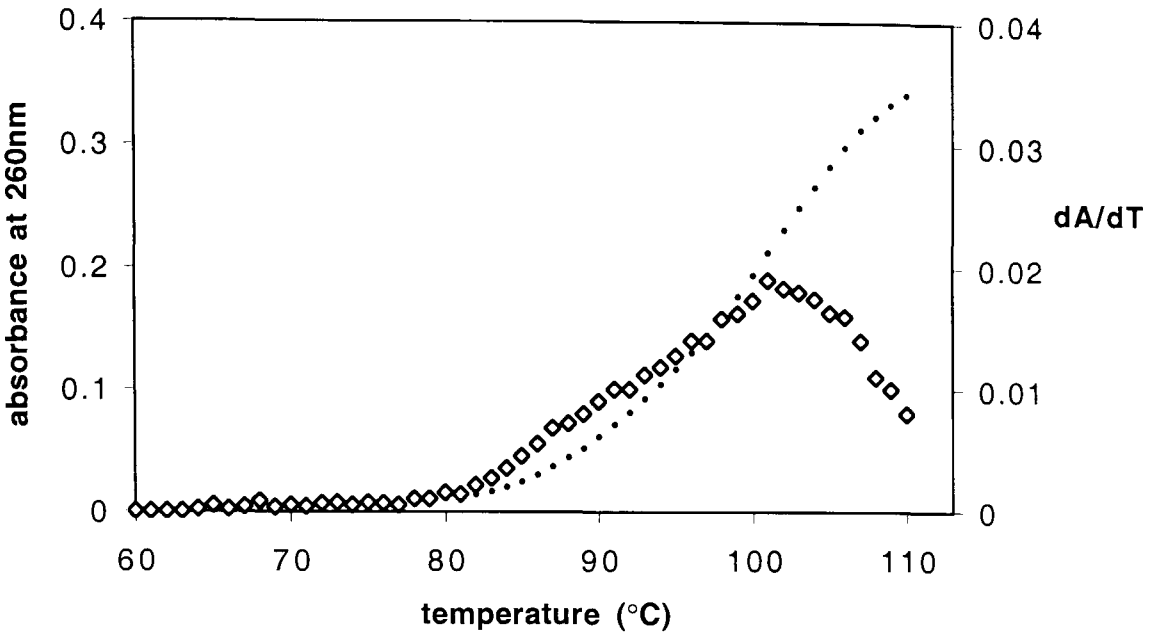


Figure 2.17: Melting profile of calf thymus DNA in the presence of AQ6, showing A vs T (•) and dA/dT vs T (◊). Values shown represent the mean of three experiments.

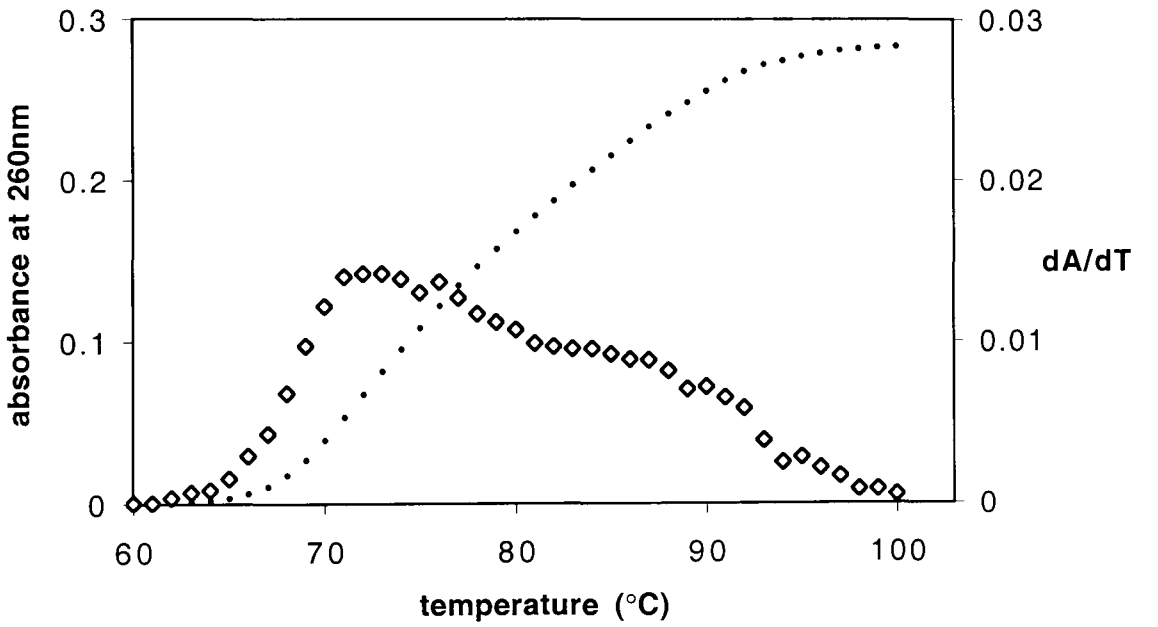


Figure 2.18: Melting profile of calf thymus DNA in the presence of AQ6N, showing A vs T (•) and dA/dT (◊). Values shown represent the mean of three experiments.

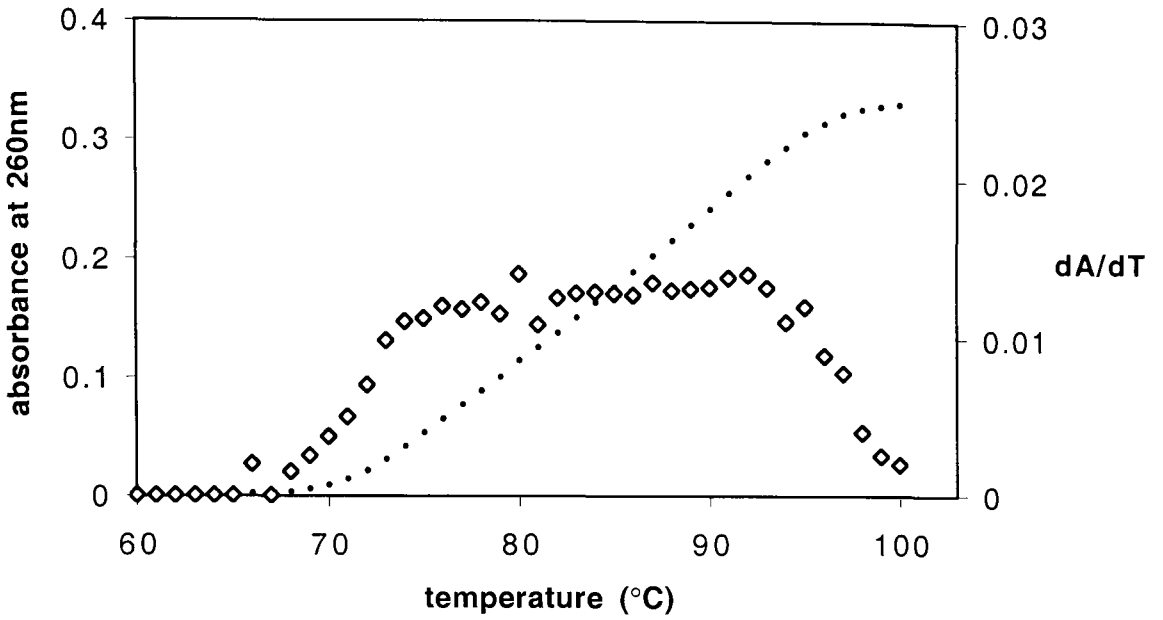


Figure 2.19: Melting profile of calf thymus DNA in the presence of AQ11, showing A vs T (•) and dA/dT vs T (◊). Values shown represent the mean of three experiments.

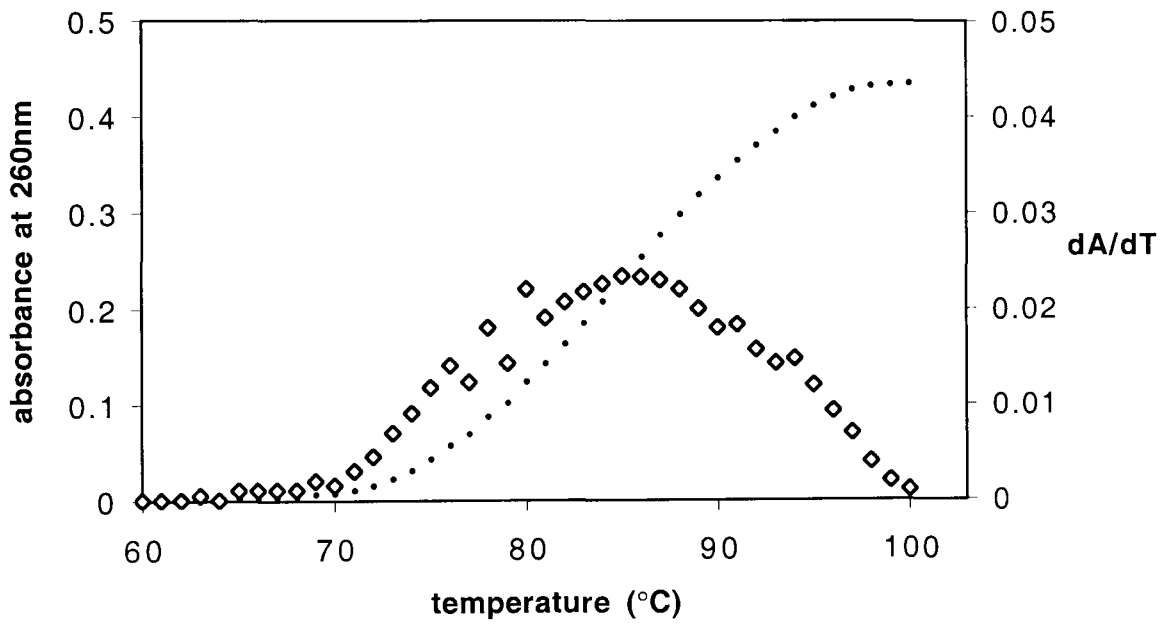


Figure 2.20: Melting profile of calf thymus DNA in the presence of AQ12, showing A vs T (•) and dA/dT vs T (◊). Values shown represent the mean of three separate experiments.

2.4 DISCUSSION

2.4.1 TOPOISOMERASE II INHIBITION STUDIES

Two methods frequently used to measure topoisomerase II activity are the decatenation assay and the DNA relaxation assay. However, assays based upon the relaxation of supercoiled DNA can be complicated by the presence of topoisomerase I and nucleases in partially purified fractions (Sahai & Kaplan, 1986). In contrast, the decatenation assay is absolutely specific for type II topoisomerase activity, because type I enzymes are unable to achieve decatenation unless at least one of the two substrate DNA molecules is nicked (Maxwell & Gellert, 1986; Barrett *et al*, 1990). The decatenation assay was thus chosen for this study as the V79 cell topoisomerase II nuclear extract may have contained low levels of topoisomerase I and/or nuclease activity. In addition, the relaxation assay was also unsuitable as the drugs screened were intercalators, which are known to unwind the DNA helix *per se*.

Mitoxantrone inhibited decatenation of kDNA at 0.75μM. Inhibition was presumably due to inhibition of topoisomerase II-catalysed DNA strand passing, resulting in stabilization of the topoisomerase II-DNA-drug cleavable complex (see section 1.3.2). AQ4 and AQ6 both inhibited topoisomerase activity at concentrations similar to mitoxantrone, and considering their structural similarity with this compound, they are also likely to stabilize cleavable complexes. However, the N-oxides, AQ4N and AQ6N, showed a marked difference to the parent compounds in their ability to inhibit topoisomerase II (table 2.1). This demonstrates that incorporation of the N-oxide functionality, which diminishes DNA intercalation (*vide infra*) also prevents inhibition of topoisomerase II.

The fact that mitoxantrone was found to be the most potent of the parent compounds may be attributed to long-term trapping of topoisomerase II cleavable complexes by this drug. The persistence of mitoxantrone-topoisomerase II cleavable complexes has been demonstrated in small cell lung cancer cells (Smith *et al*, 1990) and human fibroblast cells (Fox & Smith, 1990). The longevity of the complexes formed

could relate to its DNA-drug binding mode (see section 2.4.2) or may involve one or more active metabolites of the drug (see section 1.2.3). The 2'-(hydroxyethylamino)-ethylamino side chain of mitoxantrone is known to be metabolized to produce the corresponding mono- and dicarboxylic acid derivatives, detected in human hepatocytes (Richard *et al*, 1991) and human plasma and urine samples (Ehninger *et al*, 1986; Schleyer *et al*, 1994). These metabolites have been shown to be inactive (Chiccarelli *et al*, 1986), but it is likely that oxidation of the alcohol groups proceeds via an aldehyde intermediate, as oxidation from an alcohol to an acid can not be accomplished in a single step (Fessenden & Fessenden, 1986). The aldehyde intermediates may achieve cleavable complex stabilization via their ability to form Schiff's bases with nucleophilic sites on topoisomerase II (figure 2.21; also see figure 5.24, section 5.4.2). In the presence of water, these are reversible, but the DNA-topoisomerase II active site appears to be hydrophobic (Capranico *et al*, 1997). Thus, Schiff's base formation may result in persistently trapped topoisomerase II cleavable complexes in the hydrophobic interior of the topoisomerase II-DNA active site. In support of this, cell lines resistant to mitoxantrone have been found to contain a reduction in tightly bound, non-exchangeable drug (Beck *et al*, 1987; Pincus & Goldman, 1990). In addition, irreversible cleavable complex stabilization has been demonstrated for other topoisomerase II inhibitors (Kawada *et al*, 1991) and topoisomerase I inhibitors (Hertzberg *et al*, 1990).

A third metabolite which has been detected in urine and serum (Ehninger *et al*, 1986) could conceivably correspond to an aldehyde derivative of mitoxantrone. In support of this, the metabolite is more polar than mitoxantrone, but less polar than either of the carboxylic acid derivatives. It is also comparatively short-lived, which would be expected of an aldehyde in view of their highly reactive nature and rapid oxidation in biological systems (Beedham, 1997). In this study, activation to produce the aldehyde intermediate of mitoxantrone could have been mediated by enzymes in the V79 nuclear extract, e.g. alcohol dehydrogenase, an enzyme located notably in the soluble fraction of liver, kidney and lung cells (Bosron & Li, 1980; Gibson & Skett, 1986).

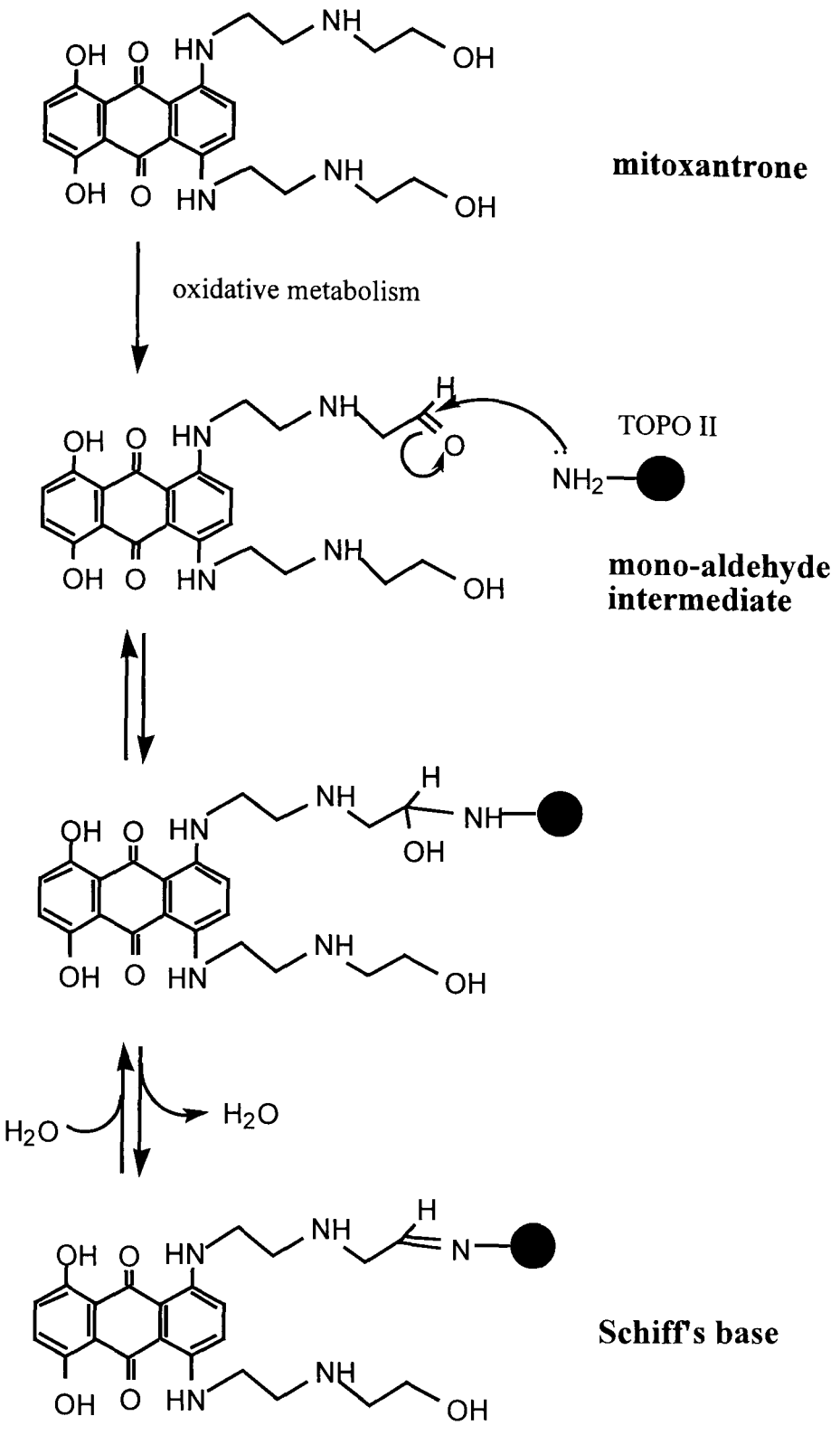


Figure 2.21: Proposed mechanism of Schiff's base formation between the mono-aldehyde intermediate of mitoxantrone and topoisomerase II. Formation of the di-aldehyde intermediate may lead to Schiff's base formation on both side chains.

2.4.2 DNA BINDING STUDIES

Altering the nature of both the chromophore and side chains of the anthraquinones was found to affect their DNA binding properties. The most significant finding was that modification of the terminal nitrogen of the alkylamino side chain to form an N-oxide resulted in a large decrease in DNA binding affinity. This demonstrates that the electrically neutral N-oxide functionality prevents stabilization of intercalative binding normally associated with cationic alkylaminoanthraquinones. This is attributable to a reduction in the electrostatic attraction between amino groups of the side chains of the drug and the negatively charged PO_4^- groups of the DNA backbone. As the mono N-oxide AQ6N possessed one cationic alkylamino side chain, some DNA binding activity was retained.

All parent compounds displayed batho- and hypochromic shifts in the visible region of the spectrum and a large majority displayed an isosbestic point at a concentration of 0.5M NaCl. However, for some compounds, there was no clear isosbestic point at a concentration of 0.05M NaCl. This is consistent with the previous observation that mitoxantrone loses isosbestic properties below a concentration of 0.1M NaCl, particularly at higher DNA to drug ratios (Lown *et al*, 1985, Kapuscinski & Darzynkiewicz, 1985). This has also been observed for daunomycin-DNA complexes (Chaires *et al*, 1982). It would appear that at lower ionic strengths, there are various drug forms in equilibrium in solution due to other DNA-drug binding modes (Monnot *et al*, 1991; figure 2.22). Firstly, the drug molecule can bind to the exterior of the DNA helix. This involves electrostatic interaction of the alkylamino side chains with the DNA phosphate backbone. Secondly, when the exterior of the DNA helix becomes saturated, further drug molecules can associate with those which are already surface bound via hydrophobic-hydrophobic interactions, in a process referred to as "stacking".

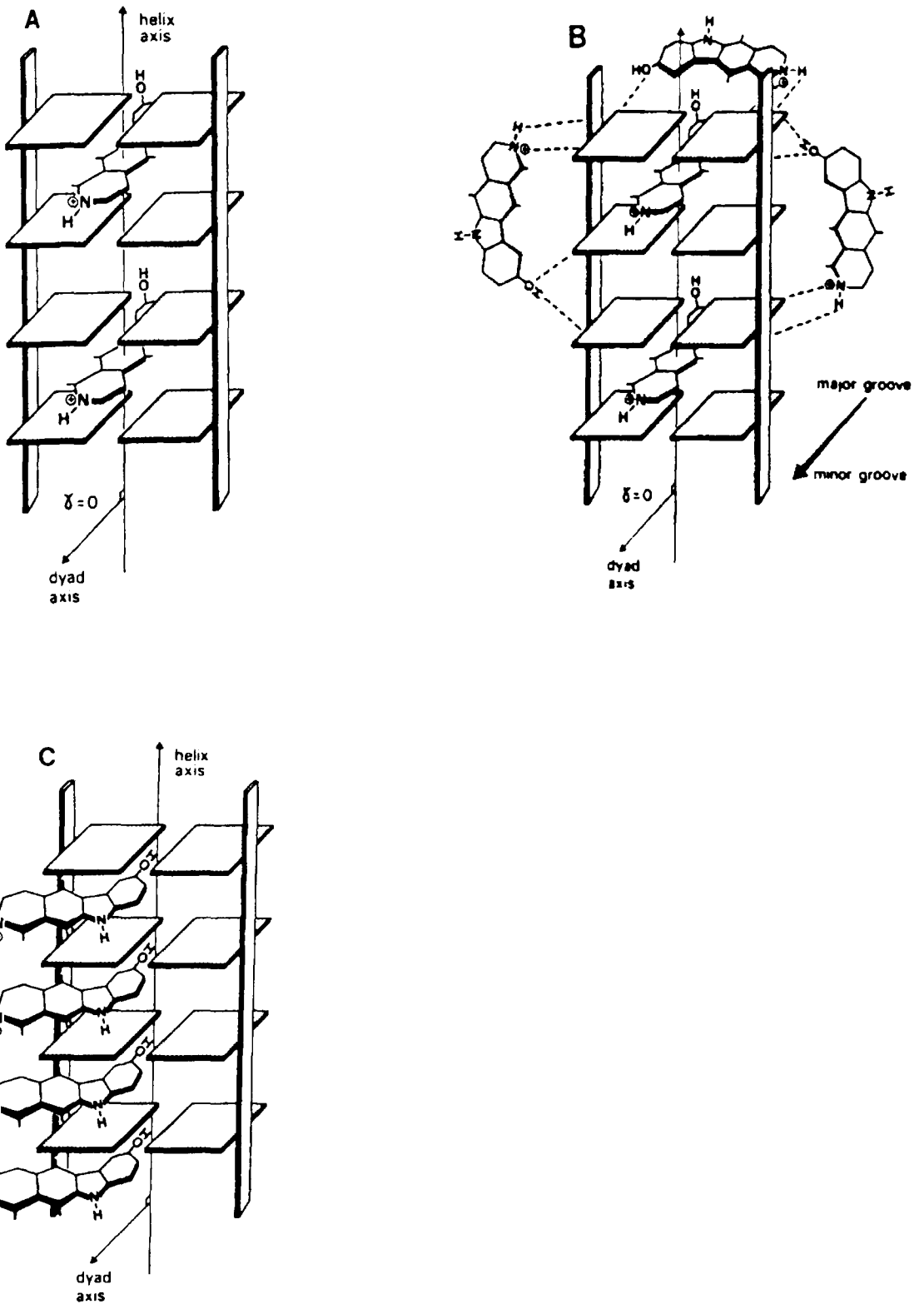


Figure 2.22: Three site binding model showing (A) intercalation, (B) external binding and (C) self association for ellipticine derivatives with poly[d(A-T)]. Bis-substituted alkylaminoanthraquinones such as mitoxantrone probably display similar behaviour.

Surface binding and/or stacking would account for the fact that the isosbestic point was lost particularly at lower DNA:drug ratios, where there would be limited intercalation sites available for drug binding. Clear isosbestic points were seen at higher ionic strengths (0.5M) because the DNA surface was neutralized by Na^+ ions, preventing electrostatic DNA-drug interactions even at higher drug concentrations (see Jones *et al*, 1980).

The fact that the disappearance of the isosbestic point was more evident in the compounds containing both side chain and chromophore hydroxy groups (particularly AQ11, which contains six OH groups) would suggest that surface binding and stacking are encouraged by these groups. Surface binding may result from hydrogen bonding between the side chain hydroxyl groups of the drug and the phosphate oxygen groups of DNA (Pohle *et al*, 1990). The presence of hydroxy groups at the 5 and 8 positions of the chromophore appears to influence their degree of stacking by encouraging the formation of dimers and perhaps higher aggregates. This has been demonstrated with mitoxantrone, which has more than a 4-fold higher dimerization constant than ametantrone (Kapuscinski & Darzynkiewicz, 1985). Surface binding and stacking have both been suggested as reasons for the difference in potency of the two drugs (see section 1.2.2). However, as only the monomer should be taken into consideration in the intercalation process as proposed by Lerman (1961), an isosbestic point was essential for the determination of K and n.

All compounds were found to have DNA binding constants in the 10^6 M^{-1}range, consistent with intercalation as the mode of DNA binding (Kapuscinski *et al*, 1981; Kapuscinski & Darzynkiewicz, 1985; Lown *et al*, 1985). At the ionic strength under which the K and n values were determined, external binding of the ligand to sugar-phosphate backbone was effectively precluded, so that intercalation was the only binding mode. It was anticipated that this may have a small lowering effect on the binding constant due to reduced interaction of the alkylamino side chains of the

intercalated drug with the DNA phosphate backbone. Overall, the K values for ametantrone and its congeners were found to be lower than for mitoxantrone and its congeners, indicating a diminished affinity for DNA, associated with the absence of hydroxy groups at the 5 and 8 positions. However, the K value for ametantrone was higher than for the other non-hydroxylated analogues, AQ12 and AQ16, which can probably be attributed to the presence of the 2'-(hydroxyethylamino)-ethylamino side chains. Other than this, variations in the groups terminal to the aliphatic amino group of alkylamino side chains did not significantly affect binding to DNA, as long as the cationic nature was maintained. This has previously been found to be the case with respect to the dissociation rates of alkylaminoanthraquinone-DNA complexes even when the alterations in side chain structure are relatively large, for example, introduction of morpholino or other groups terminal to the aliphatic amino group (Denny & Wakelin, 1990).

The different K values obtained for mitoxantrone and ametantrone and their respective analogues may relate to differences in their binding kinetics. As K is an equilibrium constant, it will be affected by the rate at which drug molecules associate with, and dissociate from, DNA. While no significant difference has been found between the association rates of drugs such as mitoxantrone and ametantrone with DNA, the 5,8 hydroxy groups of the mitoxantrone chromophore have been found to contribute to a three- to four-fold retardation in the overall dissociation process compared to ametantrone (Lown *et al*, 1985; Denny & Wakelin, 1990). Thus, it could be argued that the slower dissociation rate of the 5,8-dihydroxylated analogues from DNA is responsible for their higher K values. It has been proposed that the release of mitoxantrone from the drug-DNA complex is hindered by the bulkiness of the 5,8 hydroxy groups, which protrude through the helix almost to the opposite groove, and therefore must pass through the helix as the drug disengages (Denny & Wakelin, 1990) Furthermore, intramolecular hydrogen bonding between the 5,8 dihydroxy groups and the carbonyl groups of the anthraquinone ring, by increasing the overall lipophilicity of

mitoxantrone compared to ametantrone, will prolong retention of the mitoxantrone chromophore in the hydrophobic interior of the double helix (Patterson, 1993). However, more recently, another explanation for the higher DNA binding affinity of mitoxantrone has been proposed using molecular dynamics simulations (Mazerski *et al*, 1998). It is surmised that due to the presence of the 5,8 OH groups, the mitoxantrone molecule is significantly wider than that of ametantrone, and as a result, lies in a more coplanar orientation within the intercalation site, increasing its interaction with the base pairs. This would make the DNA-drug complex formed by mitoxantrone more energetically favourable.

The rate of DNA-drug dissociation is likely to be important in determining the cytotoxic activity of the alkylaminoanthraquinones, as slower dissociation of drug from DNA is likely to increase the probability of disrupting essential processes such as DNA replication and transcription (Safa *et al*, 1983; Safa & Tseng, 1984) and topoisomerase II inhibition. This may account for the relatively high biological potency of mitoxantrone both *in vitro* (Evenson *et al*, 1979; Locher & Meyn, 1983) and *in vivo* (Johnson *et al*, 1979; Krapcho *et al*, 1991) compared to ametantrone. In support of this, several previous studies have indicated that slower DNA-drug dissociation correlates with an increase in antitumour activity of DNA intercalating agents (Feigon *et al*, 1984, Wakelin *et al*, 1987). Moreover, ring hydroxylation has also been shown to increase the potency of other anthraquinone-based agents, e.g. the anthrapyrazoles (Showalter *et al*,1987).

Although Kapuscinski & Darzynkiewicz (1985) have reported a higher affinity constant for ametantrone than for mitoxantrone with calf thymus DNA, these data were obtained by the reverse titration method (DNA titrated with drug) and thus can not be directly compared to the results obtained in this study. In addition, their experiments were carried out at the lower ionic strength of 0.15M NaCl. Their reasoning for reverse titration was that the existence of high DNA:drug ratios may encourage DNA condensation. However, DNA condensation was not observed in this study, perhaps due

to the high ionic strength used.

Scatchard analysis assumes independent (non-interacting) drug binding sites. However, the n values in particular may be influenced by binding of other drug molecules to DNA. The site exclusion (nearest-neighbour exclusion) model, describes a process whereby the binding of a drug molecule to one site is thought to influence the binding of subsequent molecules. In particular, subsequent drug molecules are excluded from binding at adjacent or nearby sites, either by physical blocking or steric alterations in the DNA (Crothers, 1968; McGhee & von Hippel, 1974). In addition, it is also possible that electrostatic interactions induced between the drug and DNA base pairs upon intercalation result in an alteration in charge distribution on the base pairs, such that intercalation of further molecules is less favourable. Computer graphics modeling indicates that the drug side chains are capable of forming hydrogen bonds not only with neighbouring nucleotides but also more distant ones, which would discourage binding of another drug molecule at these sites (Rehn & Pindur, 1996; Mazerski *et al*, 1998).

Site exclusion means the n value for intercalation is usually a maximum of ~0.43 drug molecules per DNA base pair, as determined from theoretical calculations. This has been verified experimentally. The n values of 0.12-0.14 obtained in this study are consistent with the site exclusion model, but are lower than those obtained by Kapuscinski & Darzynkiewicz (1985) and Lown *et al* (1985). One explanation for this is that while K values are more accurate at higher DNA:drug ratios, the value of n will be more accurate at lower DNA:drug ratios. However, both K and n were calculated at the higher DNA:drug ratios (figures 2.7 to 2.10). Calculation of n at lower DNA:drug ratios gave n values ~0.2-0.3 (results not shown).

All parent compounds were found to stabilize calf thymus DNA against thermal denaturation, as previously observed for mitoxantrone and ametantrone (Johnson *et al*, 1979). In agreement with their higher K values, the 5,8 dihydroxylated anthraquinones caused greater increases in Tm than the non-hydroxylated compounds, indicating their

greater affinity for DNA. The slightly lower Tm values of AQ11 and AQ12 compared to the other compounds may be due to the more bulky nature of the side chains, each containing four OH groups, which may cause steric hindrance at the intercalation site. In contrast to its parent compound, AQ4N caused less than a 2°C rise in Tm. This was probably due to lack of interaction of the alkylamino side chains with the DNA phosphate groups, preventing intercalation and thus stabilization of the melting process. As expected, AQ6N, where one cationic alkylamino side chain was retained, showed moderate increase in Tm.

Generation of symmetrical first derivative plots for AQ11 and AQ12 was indicative of a uniform stabilization process. However, where first derivative plots were biphasic in nature (showing two absorbance maxima), this suggested a more complex denaturation process. Biphasic denaturation curves have previously been observed by Patel & Canuel (1978) for poly(dA-dT)-daunomycin complexes, and by Chaires *et al* (1982) for daunomycin-DNA complexes. Patel & Canuel have shown that the first of the two phases results from melting of DNA regions free of drug and that the second phase of melting occurs in the regions where the drug is bound to DNA. Alternatively, the biphasic melting of DNA in the presence of the alkylaminoanthraquinones may have resulted from faster dissociation of drug from A-T-rich sites than G-C-rich sites due to an increased rate of opening of A-T sites (Malhotra & Hopfinger, 1980). The reason for some drugs producing biphasic curves and others not is unclear, but it appears to be a function of side chain rather than chromophore variation as AQ11 and AQ12, which possess identical side chains at the 1 and 4 positions of the anthraquinone chromophore, were the only two parent compounds not to display this behaviour.

In summary, the nature of the chromophore and side chains of the alkylaminoanthraquinones was seen to influence both their DNA binding affinity and their involvement in topoisomerase II inhibition. Overall, the 5,8 dihydroxy compounds had increased DNA binding affinity compared to their non-hydroxylated analogues.

This is presumably due to the prolonged retention of the drug chromophore in the hydrophobic interior of the DNA helix. Modification of the terminal nitrogen on the alkylamino side chains to form an N-oxide resulted in a large decrease in DNA binding affinity and topoisomerase II inhibition. This demonstrates the importance of the cationic alkylamino side chain in intercalative binding and also shows that DNA intercalation is a necessary prerequisite for topoisomerase II inhibition by DNA affinic agents. The presence of the two 2'-(hydroxyethylamino)ethylamino side chains of mitoxantrone enhanced its ability to inhibit topoisomerase II. This may be due to oxidation of the side chains to produce aldehyde intermediates which increase the longevity of the topoisomerase II-DNA cleavable complex by Schiff's base formation with the enzyme. This is investigated further in chapters 4 and 5.

The discovery that subtle changes in drug structure can be used to alter drug activity suggested that it would be possible to synthesize other alkylaminoanthraquinones with modified side chains, which similar to the N-oxides, would show diminished activity compared to the parent compounds. In view of the increased potency inferred upon mitoxantrone by the presence of the 2'-(hydroxyethylamino)-ethylamino side chains, a series of acetalanthraquinones was synthesized (see chapter 3). These compounds were designed with the rationale that they could be converted to their respective aldehydes, similar to the aldehyde intermediates believed to be generated in the conversion of mitoxantrone to its mono- and dicarboxylic acids. In theory, the conversion of the acetalanthraquinones to aldehydes should increase their cytotoxic activity due to their propensity to undergo nucleophilic substitution to form Schiff's bases with intracellular targets.

CHAPTER 3: SYNTHESIS OF ACETAL- AND METHOXYMORPHOLINO- ANTHRAQUINONES

3.1 INTRODUCTION

The aim of the synthetic work was to make a series of compounds which could subsequently be converted, either chemically or metabolically, to aldehydes, similar to the proposed aldehyde intermediate of mitoxantrone. Two amines incorporating *O*-methyl groups were synthesized and substituted onto the anthraquinone moiety to form 1-, 1,4-, and 1,5-substituted anthraquinones.

3.2 METHODS AND MATERIALS

Diphenylphosphinamide, *N*-[2-(2'-methoxymorpholinyl)]ethanol and 2,3-dihydro-1,4,5,8-tetrahydroxy-anthracene-9,10-dione were synthesised as described in appendix A3. All other chemical reagents were supplied by Sigma Chemical Co., Poole, Dorset, UK, and Aldrich Chemical Co., Gillingham, Dorset, UK, and were 98-99% pure. Silica gel 60 (20-45 microns) was purchased from Fisons, Loughborough, UK.

Chromatography columns (~7cm diameter x 25 cm length) were packed with 20 cm of silica. Methanol was dried with magnesium turnings, and CH_2Cl_2 was dried with CaH_2 prior to use to remove any traces of water. Ultra violet and visible spectra were recorded on a Beckman DU70 spectrophotometer, infra-red spectra were recorded on a Nicolet 205 FTIR spectrophotometer and nuclear magnetic resonance spectra were recorded on a Brucker AC250 NMR spectrometer at 250MHz. Mass spectra were performed at the Chemistry Department, University College of Swansea, Singleton Park, Wales and micro-elemental analysis was carried out at the Department of Chemistry, University of Manchester, UK. Melting temperatures were determined using Gallenkamp melting apparatus (Gallenkamp, Loughborough, UK). Amines were stored as methanolic solutions at 4°C. Acetalanthraquinones were stored *in vacuo* over P_2O_5. Where abbreviations have been used for reagents, full names can be found in appendix A8.

3.2.1 SYNTHESIS OF ALKYLAMINO SIDE CHAINS

3.2.1.1 SYNTHESIS OF 2-[*N'*-(2,2-DIMETHOXYETHYL)AMINO]ETHYLAMINE

Bromoacetaldehyde dimethylacetal, 34g (0.2 mol), was added dropwise to diaminoethane 120g (2.0 mol) over 1 h, such that the temperature did not exceed 70°C. The mixture was heated for a further 2-3 h, until the reaction was complete (monitored by thin layer chromatography in MeOH/NH_4OH 9:1), cooled to room temperature and distilled at 0.5mmHg to remove unreacted diaminoethane. The HBr salt of diaminoethane was precipitated from the solution by the addition of CH_2Cl_2, and separated from the product by filtration *in vacuo.* The CH_2Cl_2 was removed by rotary film evaporation and the product distilled at 2mmHg, collecting the fraction between 72 and 74°C. The distillate was stored at 4°C. The yield was 14.0g (47%).

M.S. 118 (M-30), 86 (M-62), 75 (M-73), 73 (M-75), 58 (M-90), 56 (M-92), 44 (M-104)

NMR 2.7 (m, CH_2 groups, 6H), 3.4 (s, -OCH_3, 6H), 4.4 (t, -CH, 1H).

BP (°C) 72-74 at 2mmHg

3.2.1.2 SYNTHESIS OF *N*-[2-(2'-METHOXYMORPHOLINYL)ETHYL]AMINE

A solution of 21g (0.18mol) methanesulfonyl chloride, in 100 ml benzene, was added over 1 h to a refluxing mixture of 25.9g diphenylphosphinamide (0.12 mol), 29g *N*-[2-(2'-methoxymorpholinyl)]ethanol (0.18 mol), 29g finely crushed NaOH (0.72 mol), 50g K_2CO_3 (0.36 mol), 8.2g tetra-*n*-butylaminium hydrogen sulfate (0.024 mol) and benzene (300 ml), and then stirred for a further 1 h at reflux. The mixture was cooled to room temperature, diluted with 150ml benzene and separated. The solid phase was dissolved in 350ml water and extracted with 350ml benzene. The benzene used for the extraction was combined with the organic phase and washed with water (4x100ml) until neutral (tested with universal indicator paper), then dried over Na_2SO_4 for 12 h. The benzene was removed by evaporation *in vacuo* and the resulting residue dissolved in tetrahydrofuran and bubbled with dry HCl gas for 15 min until the amine

hydrochloride precipitated. The THF was removed by evaporation *in vacuo* and the solid redissolved in methanol for storage.

The free base was prepared by removing the methanol by evaporation *in vacuo*, dissolving the solid in 5% NaOH and extracting the amine into CH_2Cl_2, removing the solvent by evaporation *in vacuo*. The yield was approximately 6g (crude product).

3.2.2 SYNTHESIS OF ACETAL- AND MORPHOLINOANTHRAQUINONE DERIVATIVES

3.2.2.1 SYNTHESIS OF 1-*N*-{2-[*N'*-(2,2-DIMETHOXYETHYL)AMINO]-ETHYLAMINO}-ANTHRACENE-9,10-DIONE (YCG7)

To 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine, 3g (0.02 mol), was added 1g 1-chloroanthraquinone (0.04 mol), heated so that the temperature did not exceed 50°C. On disappearance of the starting material, monitored by thin layer chromatography (100% methanol), 50 ml of methanol was added and the components of the mixture were separated by flash column chromatography on silica gel (100% MeOH). Crude product was stored in methanol at 4°C for 24 h. The recrystallized product was filtered *in vacuo*, washed with methanol (2 x 100ml) and dried over P_2O_5 *in vacuo*. The yield was 0.9g (58%).

M.P (°C) 94-95

TLC Rf (CH_2Cl_2/MeOH 9:1) 0.79

IR (cm^{-1}) 3300 (-NH), 2800-3000 (CH_2), 1670 (quinone carbonyl), 1630 (quinone carbonyl, hydrogen bonded), 1600 (aromatic CH)

^{1}HNMR (ppm) ($CDCl_3$) 2.8 (d, - CH_2, 2H), 3.0 (t, - CH_2, 2H), 3.4 (s, -O CH_3, 6H), 3.5 (q, - CH_2, 2H), 4.5 (t, -CH, 1H), 7.0-8.3 (m, aromatic -CH, 7H), 9.8 (t, aromatic -NH, 1H).

M.S 355 (M+1), major fragments at 292 (M-63), 238 (M-117), 118

CHN C 68.1, H 6.2, N 7.9. Calculated for $C_{20}H_{22}N_2O_4$ C 67.8, H 6.3, N 7.9.

UV/vis (in MeOH) vis λmax 496nm (E= 8334$M^{-1}cm^{-1}$). UV λmax 242 nm (E=37620$M^{-1}cm^{-1}$)

3.2.2.2 SYNTHESIS OF 1,4-BIS-*N*-{2-[*N'*-(2,2-DIMETHOXYETHYL)AMINO]-ETHYLAMINO}-ANTHRACENE-9,10-DIONE (YCG8)

To 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine, 6g (0.04 mol), was added 1g 2,3-dihydro-1,4-dihydroxyanthracene-9,10-dione (0.004 mol). The mixture was heated under argon, so that the temperature did not exceed 50°C. When the reaction had gone to completion, followed by thin layer chromatography (CH_2Cl_2/MeOH 4:6) the mixture was re-oxidized by stirring in air for 12 h. The reaction mixture was subjected to flash column chromatography on silica gel (CH_2Cl_2/MeOH 4:6) and the major blue fraction was collected. After removing the solvents by rotary film evaporation, the resulting solid was re-chromatographed under the same conditions as before. The major blue fraction was again collected, the solvents removed by rotary film evaporation. The solid dried over P_2O_5 *in vacuo*. The yield was 1g (47%).

M.P (°C) 60-61

TLC Rf (CH_2Cl_2/MeOH 9:1) 0.44

IR (cm^{-1}) 3300 (-NH), 2800-3000 (CH_2), 1610 (quinone carbonyl, hydrogen bonded), 1590 (aromatic CH)

^{1}HNMR (ppm) ($CDCl_3$) 2.8 (d, - CH_2, 4H), 3.0 (t, - CH_2, 4H), 3.4 (s, -O CH_3, 12H), 3.5 (m, - CH_2, 4H), 4.5 (t, -CH, 2H), 7.7 (m, aromatic H, 3H), 8.3 (m, aromatic H, 3H), 10.8 (t, -NH, 2H).

M.S 501 (M+1), major fragments at 437 (M-63), 383 (M-117), 319 (M-180), 264 (M-236), 118

CHN C 62.4, H 7.4, N 11.2. Calculated for $C_{26}H_{36}N_4O_6$ C 62.4, H 7.3, N 11.2

UV/vis (in MeOH) vis λmax 636nm (E=17423$M^{-1}cm^{-1}$). UV λmax 254nm (E=31427$M^{-1}cm^{-1}$).

3.2.2.3 SYNTHESIS OF 1,5-BIS-*N*-{2-[*N'*-(2,2-DIMETHOXYETHYL)AMINO]-ETHYLAMINO}-ANTHRACENE-9,10-DIONE (YCG9)

To 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine, 5.5g (0.037 mol), was added 1,5-di-chloroanthraquinone, 1g (0.0036 mol) and the mixture heated in air such that the temperature did not exceed 50°C. On disappearance of the starting material, followed by thin layer chromatography (CH_2Cl_2/MeOH 4:6), the reaction mixture was separated by flash column chromatography on silica gel (CH_2Cl_2/MeOH 4:6). The major pink fraction was collected and after removing the solvents by rotary film evaporation, the resulting solid was re-chromatographed under the same conditions as before. The major pink fraction was again collected, the solvents removed by rotary film evaporation and the product was dried over P_2O_5 *in vacuo*. The yield was 0.8g (42%).

M.P (°C) 79-81

TLC Rf (CH_2Cl_2/MeOH 9:1) 0.57

IR (cm^{-1}) 3300 (-NH), 2800-3000 (CH_2), 1620 (quinone carbonyl, hydrogen bonded), 1600 (aromatic CH)

^{1}HNMR (ppm) ($CDCl_3$) 2.8 (d, - CH_2, 4H), 3.0 (t, - CH_2, 4H), 3.4 (s, -O CH_3, 12H), 3.5 (q, - CH_2, 4H), 4.5 (t, -CH, 2H), 7.0 (d, aromatic H, 2H), 7.5 (m, aromatic H, 4H), 9.8 (t, -NH, 2H).

M.S 501 (M+1), major fragments at 437 (M-63), 383 (M-117), 319 (M-180), 265 (M-235), 118

CHN C 59.8, H 7.3, N 10.9. Calculated for $C_{26}H_{36}N_4O_6.1.25\ H_2O$ C 59.7, H 7.4, N 10.7

UV/vis (in MeOH) vis λmax 516nm (E=11360$M^{-1}cm^{-1}$). UV λmax 230nm (E= 38400$M^{-1}cm^{-1}$).

3.2.2.4 ATTEMPTED SYNTHESIS OF 1-*N*-{2-[*N'*-(2,2-DIMETHOXYETHYL)-AMINO]ETHYLAMINO}-5,8-DIHYDROXY-ANTHRACENE-9,10-DIONE (YCG5)

A solution of 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine, 1g (0.0068 mol), in methanol, was added to 1g of 2,3-dihydro-1,4,5,8-tetrahydroxyanthracene-9,10-dione (0.0037 mol) under argon, and the mixture left to react at room temperature for 4 h. The mixture was then separated by flash column chromatography on silica gel (CH_2Cl_2/MeOH 19:1). The fraction containing the major purple band was collected and the solvents removed by high vacuum distillation at 21°C. The solid was chromatographed again, under the conditions described above, and the major purple component collected. After removing the solvents by high vacuum distillation at 21°C, the compound was dried over P_2O_5 *in vacuo*.

3.2.2.5 ATTEMPTED SYNTHESIS OF 1,4-BIS-*N*-{2-[*N'*-(2,2-DIMETHOXY-ETHYL)AMINO]ETHYLAMINO}-5,8-DIHYDROXY-ANTHRACENE-9,10-DIONE (YCG6)

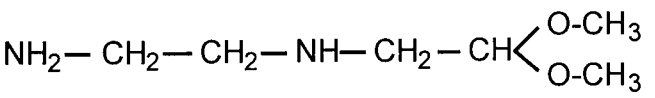
To 11g 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine (0.074 mol) under argon, was added 1g of 2,3-dihydro-1,4,5,8 tetrahydroxyanthracene-9,10-dione (0.0037 mol) and left to react in the dark for 24 h at 21°C. The resulting mixture was then subjected to flash column chromatography on silica gel (CH_2Cl_2/MeOH 19:1). The eluent containing the major blue band was collected and the solvent removed by high vacuum distillation at 10°C. The resulting material was further separated by flash column chromatography on silica gel (CH_2Cl_2/MeOH 19:1). The major blue band was retained and the solvents removed as before. The resulting solid was dried over P_2O_5 *in vacuo* and stored at -15°C in the dark.

3.2.2.6 ATTEMPTED SYNTHESIS OF 1,4-BIS-*N*-{[2-(2'-METHOXY-MORPHOLINYL)ETHYL]AMINO}-5,8-DIHYDROXY-ANTHRACENE-9,10-DIONE (YCG4)

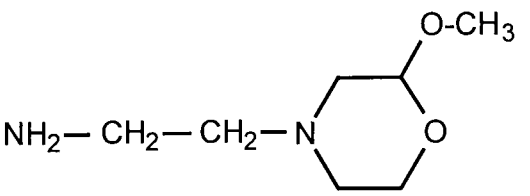
To 1g 2,3-dihydro-1,4,5,8-tetrahydroxyanthracene-9,10-dione under argon (0.0037 mol), was added 5.8g (0.036 mol) *N*-[2-(2'-methoxymorpholinyl)ethyl]amine, and left to react at 50°C for 12 h. The mixture was separated by flash column chromatography on silica gel (CH_2Cl_2/TEA 99:1). The major blue band was collected and the solvents removed by rotary film evaporation. The resulting solid was re-chromatographed on silica gel (CH_2Cl_2/ TEA 99:1). The major blue fraction was retained, the solvent removed by rotary film evaporation and the solid dried over P_2O_5 *in vacuo*. The yield was 42mg (2%).

3.3 RESULTS AND DISCUSSION

3.3.1 SYNTHESIS OF ALKYLAMINO SIDE CHAINS



2-[*N*'-(2,2-dimethoxyethyl)amino]ethylamine



***N*-[2-(2'-methoxymorpholinyl)ethyl]amine**

By design, the amines synthesized were potential substrates for acid-catalyzed hydrolysis, and thus certain precautions had to be taken during their preparation to protect the acetal groups. It was absolutely essential to dry all solvents, since silica gel, which was used in the separation of the acetalanthraquinones, is known to be a particularly convenient reagent for hydrolysis of acetals in aqueous environments (Huet *et al*, 1978). It was also critical to monitor the temperature at which reactions were performed in order to avoid cyclization of the products.

Reacting a large excess of diaminoethane with bromoacetaldehyde dimethylacetal achieved the synthesis of 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine in relatively good yields. The bromoacetal was susceptible to nucleophilic substitution due to the electron withdrawing effect of the Br group, rendering the carbon atom to which it is attached δ+ and therefore readily attacked by nucleophiles. The reaction proceeds via the S_N2 mechanism, with subsequent elimination of HBr (figure 3.1). A large excess of diaminoethane (x10) was used to minimize reaction of the acetal at both ends of the amine.

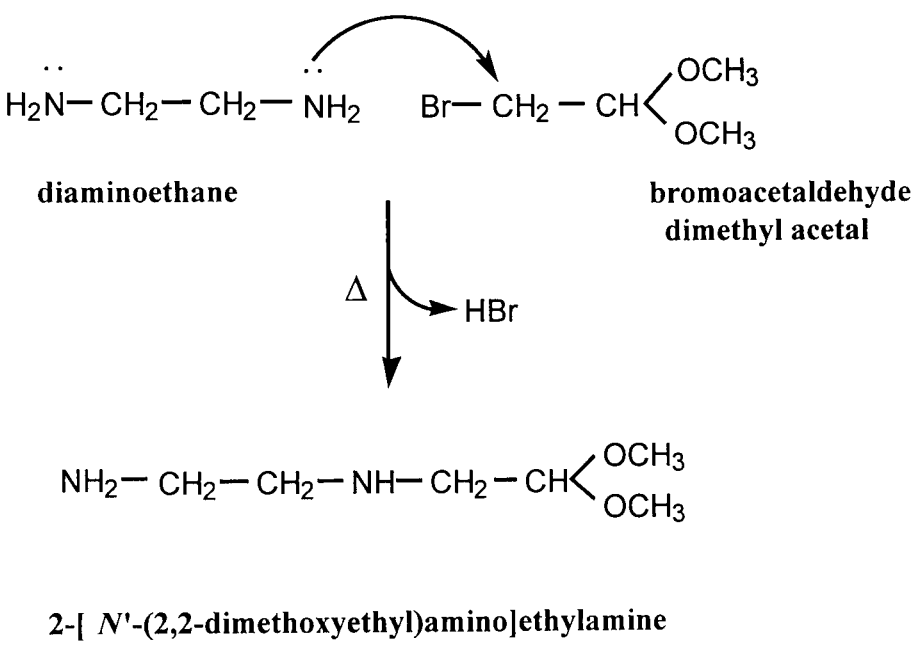


Figure 3.1: Synthesis of 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine by nucleophilic substitution of bromoacetaldehyde dimethylacetal with diaminoethane

Due to the potential of the product to undergo cyclization, the reaction was carried out below 70°C. As the reaction was exothermic, it was necessary to add the acetal to the base very slowly. The reaction was monitored by thin layer chromatography using MeOH/NH_4OH 9:1. The product, detected with iodine, had an Rf of 0.37. On completion of the reaction, excess diaminoethane was immediately removed *in vacuo* in order to reduce the basicity of the environment, since basic conditions would tend to encourage cyclization of the product by localizing the lone pair of electrons on the primary amino group nitrogen of the product. Once the diaminoethane had been removed, the principal by-product of the reaction, the hydrogen bromide salt of diaminoethane, was precipitated with dichloromethane. Attempting to precipitate the salt before evaporating diaminoethane was found to require very large amounts of solvent. This was presumably due to the partial solubility of the salt in diaminoethane. Once obtained, the crude product was distilled at 0.5mmHg and characterized by NMR, which verified its structure. The yield, approximately 47%, was probably lowered due to the evaporation of some of the product during the removal of the diaminoethane.

Synthesis of *N*-[2-(2'-methoxymorpholinyl)ethyl]amine was achieved using a method devised by Slusarska & Zwierzak (1981) for the synthesis of primary amines. This involved conversion of the morpholino alcohol to its corresponding methanesulfonate, producing a particularly convenient leaving group in the mesylate moiety. This was necessary because of the poor leaving group character of the hydroxide anion, which meant that direct conversion of the alcohol into the primary amine could not be achieved via simple nucleophilic displacement. As the methanesulfonate was immediately used for the alkylation of diphenylphosphinamide, there was no apparent need to isolate it, so it was generated *in situ* in the reaction medium. This made the method particularly convenient as it allowed generation of the amine product from the starting material in one step (figure 3.2).

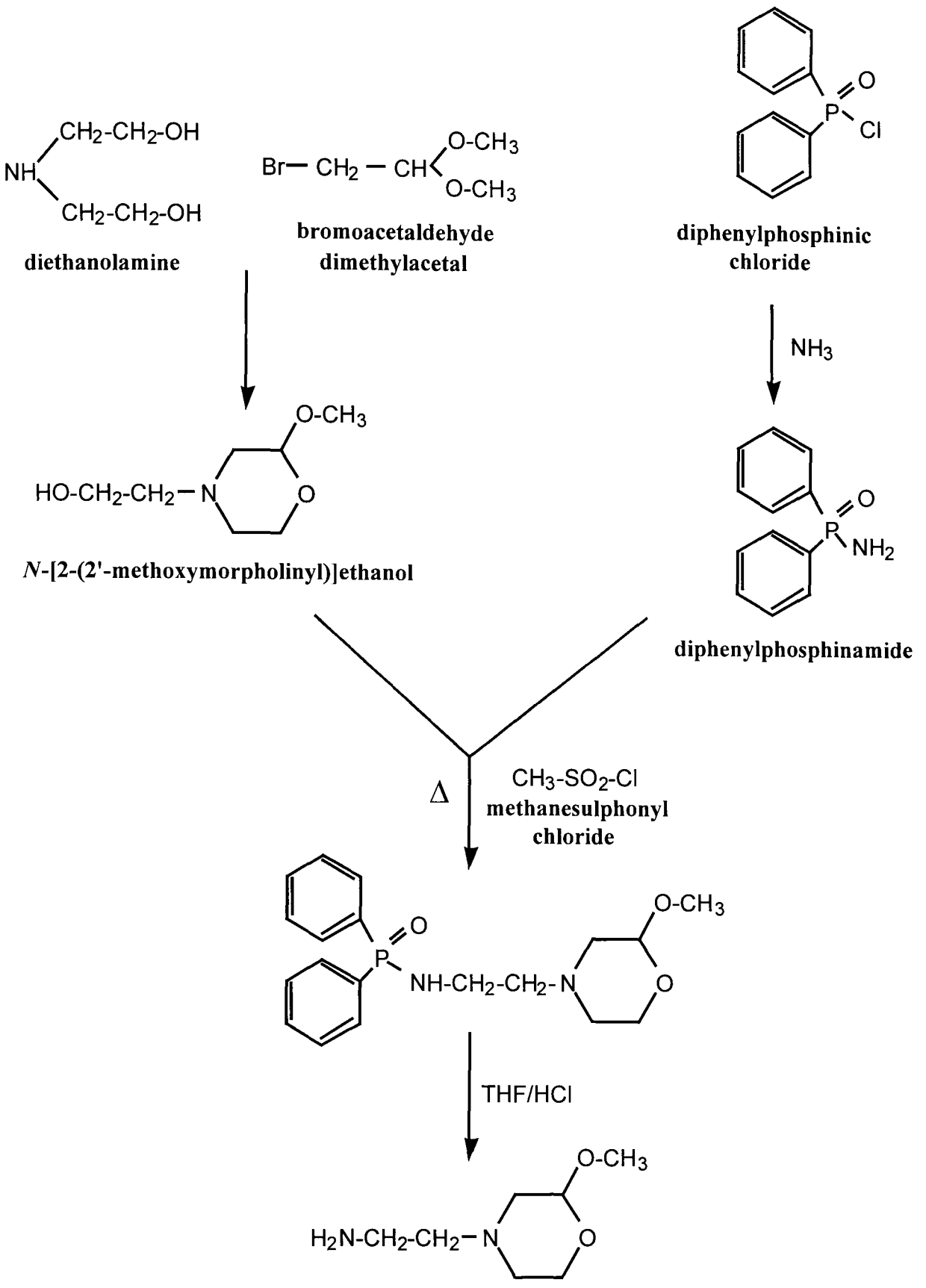
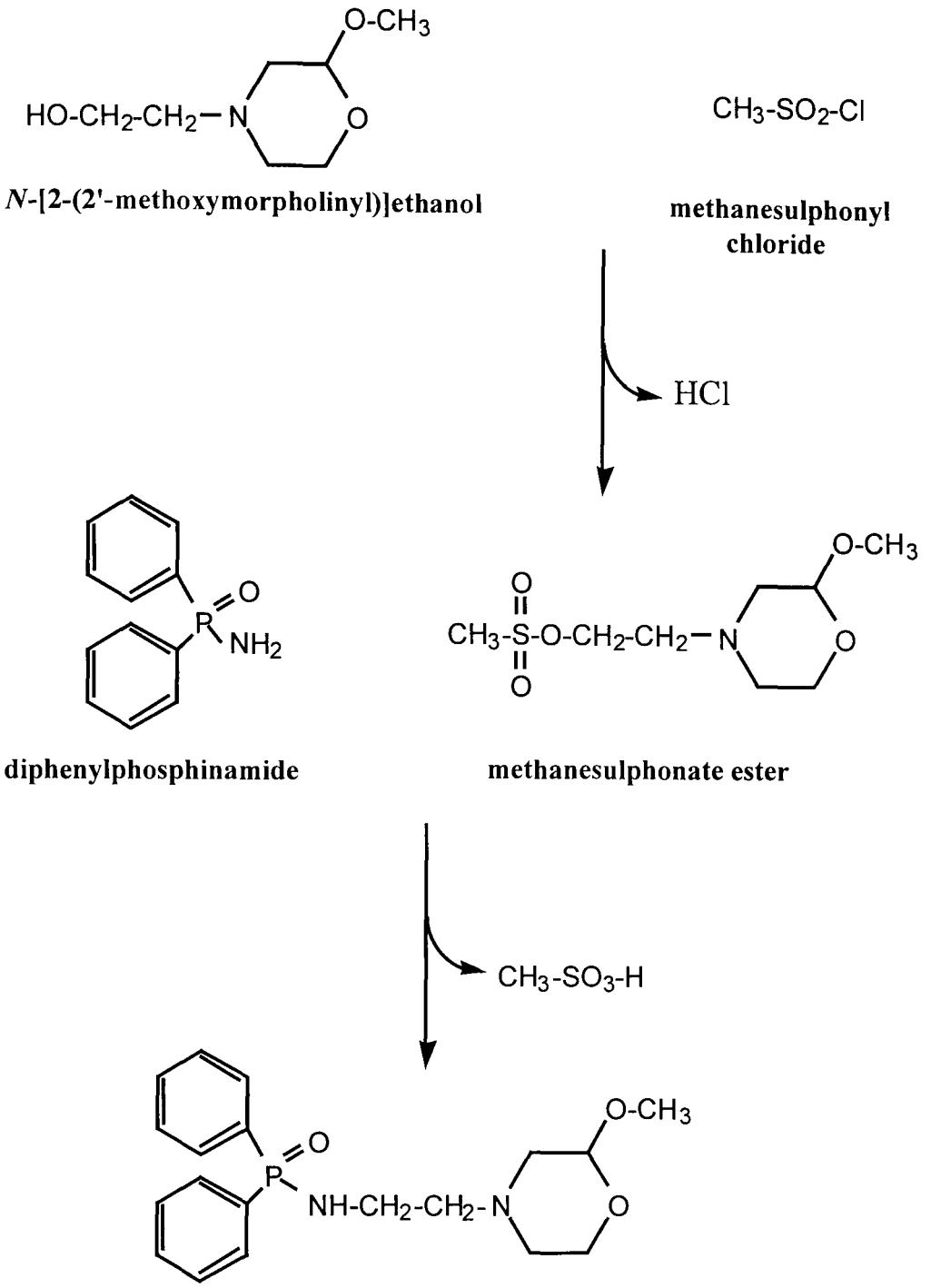


Figure 3.2: Synthesis of *N*-[2-(2'-methoxymorpholinyl)ethyl]amine by the method devised by Slusarska & Zwierzak (1981).

Due to easy deprotection of an amino function under mild conditions, degradation of the *N*-alkyldiphenylphosphinamide is usually achieved by a standard method, for example, using HCl. However, to protect the morpholino ring from acid-catalysed hydrolysis, the product was cleaved by dissolving in THF and passing dry HCl gas through the solution, generating a primary amine hydrochloride.

Advantages of using this synthetic route were that it produced only the primary amine, rather than a mixture containing secondary or tertiary amines. The *N*-alkyldiphenylphosphinamide product should have been formed in moderate yields (~50%) and high purity. However, this method resulted in a low product yield (~5%) and difficulty was encountered in attempting to separate the amine from the other reaction components. Prominent mass spectrum peaks were observed at 130 and 217 (EI) suggesting the presence of unreacted alcohol and diphenylphosphinamide respectively. Although the peak at 130 could have been derived from the amine, this seems unlikely considering the low product yield and the presence of unreacted diphenylphosphinamide. Nevertheless, a minor peak was observed at 160, indicating the presence of a small quantity of the amine product. NMR also revealed the presence of the *N*-alkyldiphenylphosphinamide.

As alkyl methanesulfonates are easily obtained from the corresponding alcohols in good yields under liquid-liquid phase-transfer catalysis conditions in benzene at room temperature (Szeja, 1979), the yield may have been improved by isolating the alkyl methanesulfonate and then adding diphenylphosphinamide to produce *N*-alkyldiphenylphosphinamide (figure 3.3).



***N*-[2-(2'-methoxymorpholinyl)]ethanol**

methanesulphonyl chloride

diphenylphosphinamide

methanesulphonate ester

***N*-alkyldiphenylphosphinamide**

Figure 3.3: Synthesis of the methanesulfonate ester of *N*-[2-(2'-methoxy-morpholinyl)]ethanol followed by *N*-alkylation to produce the *N*-alkyldiphenyl-phosphinamide.

There are also a number of alternative routes for synthesizing primary amines (see March, 1985), which were not investigated. One other route that can be used to synthesize primary amines exclusively, i.e. without the formation of secondary amines, is the Gabriel synthesis. This involves treating potassium phthalimide with an alkyl halide, which is subsequently hydrolysed to yield the product (Gibson & Bradshaw. 1968). Several other methods exist for synthesizing primary amines (see March, 1985) but like the Gabriel synthesis, these routes would have been considerably less convenient than the one used bearing in mind that the starting material was an alcohol.

3.3.2 GENERAL SYNTHESIS OF ALKYLAMINOANTHRAQUINONES

The synthesis of mono- and bis-substituted anthraquinones is based largely on aromatic nucleophilic substitution (S_Nar), generally described as the substitution of an electron withdrawing group which is attached to an aromatic nucleus by a group rich in electrons. There are two mechanisms by which nucleophilic substitution can proceed (see Fessenden & Fessenden, 1986). S_N1 reactions involve elimination of the 'leaving' group to generate a carbocation which subsequently combines with the nucleophile. S_N2 reactions, on the other hand, proceed via a bimolecular mechanism whereby the nucleophile and the leaving group attack and depart simultaneously.

The electron withdrawing property of the carbonyl groups of the anthraquinones facilitates S_N2 nucleophilic substitution of the aromatic nucleus by promoting delocalization of the positive charge across the three benzene rings (figure 3.4). For 1- and 1,5-substituted halo-anthraquinones, delocalization activates the aromatic nucleus to nucleophilic substitution by increasing the positive charge at the position on the ring to which the halogen is attached (figure 3.5). This produces monosubstituted and 1,5-bis-substituted products respectively. However, for 1,4-bis-substituted derivatives, displacement of the first halogen group is relatively easy but displacement of the second is much harder to achieve due to the electron inductive effect of the mono-amino substituent, which weakens the activating effect of the carbonyl group.

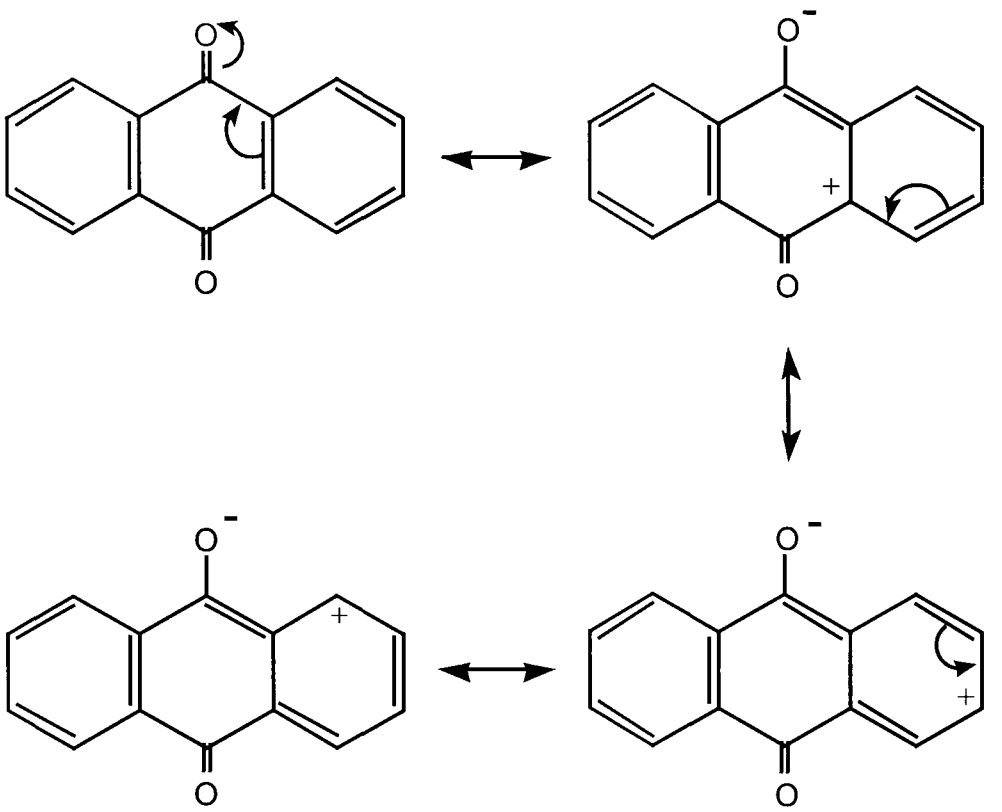


Figure 3.4: Delocalization of positive charge within the anthraquinone ring system. Delocalisation is shown across only one ring and one of the quinone carbonyl groups. Charge is distributed in a symmetrical manner across the other ring and carbonyl group.

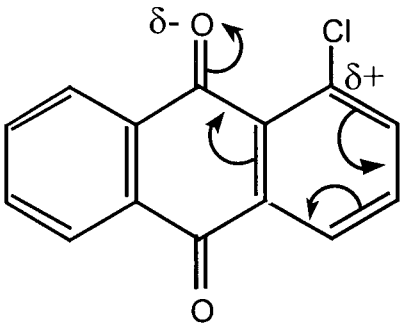


Figure 3.5: The electron withdrawing effect of the anthraquinone carbonyl group

A more productive alternative to 1,4-substituted halo-anthraquinones is quinizarin (1,4-dihydroxyanthraquinone). The reactivity of quinizarin is largely governed by its oxidation state. In its oxidized form, the electron withdrawing effect of the quinone carbonyl promotes substitution of a hydroxyl group to form the mono-substituted compound (Zee-Cheng *et al*, 1979; Kikuchi *et al*, 1982). This reaction only occurs on heating. However, on reduction, quinizarin is converted to the 2,3-dihydro (leuco) form which has been shown by NMR to assume the 1,4-dione-9,10-dihydroxy configuration (Bloom & Hutton, 1963; Kikuchi *et al*, 1981). Leucoquinizarin undergoes nucleophilic substitution with amines far more readily than the parent quinizarin, producing both monoamino and diaminoanthraquinone derivatives. The proportion of these products is dependent upon the reaction conditions (Kikuchi *et al*, 1981).

Condensation of leucoquinizarin with an excess amount of the appropriate amine generates a series of 1,4-bis-substituted anthracene-9,10-dione derivatives (Stilmer & Perkins, 1955; Simon, 1963). These products have been shown to exist in the 2,3-dihydro-1,4-bis-alkylamino-9,10-dione form, which revert to 1,4-bis-alkylamino-anthraquinones upon oxidation (figure 3.6). Stirring in air is usually sufficient to achieve oxidation of the product, although alternatives such as chloroanil (Murdock *et al*, 1979) or alkaline solution (Cheng & Zee-Cheng, 1983) have been used. The 1,4-bis-substituted anthracene-9,10-dione derivatives have a characteristic absorbance spectrum in the visible region of the spectrum with λmax values between 525 and 660nm (see section 2.3.2.2) and hence appear blue in solution. As anthraquinone starting materials usually have different absorbance spectra to their substituted products, the progression of the reaction can be monitored relatively easily using thin layer chromatography. It is also possible to determine the position of substitution on the chromophore as the colour change is characteristic of the particular substitution pattern.

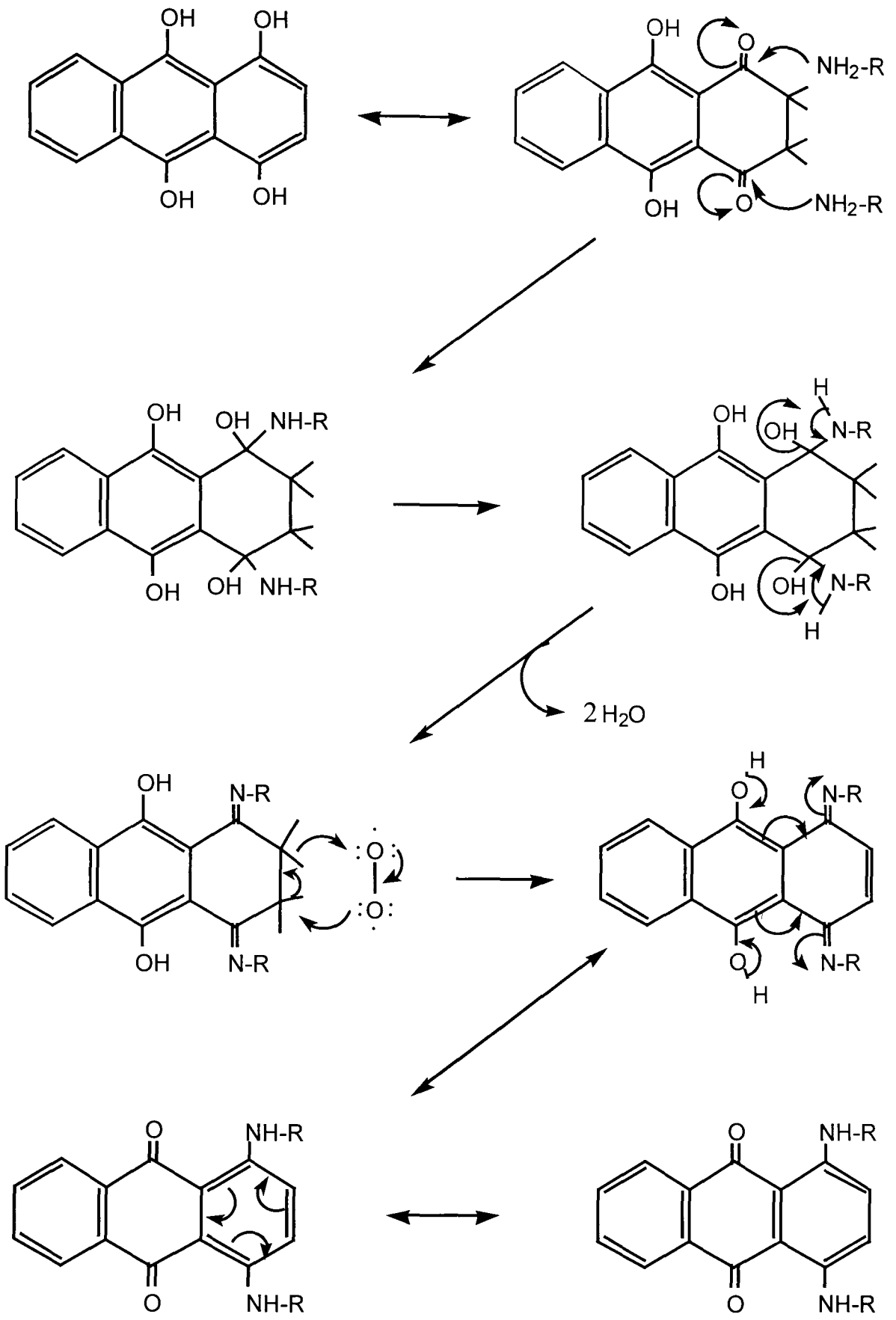


Figure 3.6: Reaction scheme showing the synthesis of 1,4-bis-substituted derivatives from 1,4-dihydroxyanthraquinone. A similar mechanism operates for the formation of 1- and 1,4-substituted compounds from 1,4,5,8-tetrahydroxy-anthraquinone.

Mitoxantrone can be synthesized from the dihydro form of 1,4,5,8-tetrahyroxy-anthracene-9,10-dione (5,8-dihydroxy-quinizarin) and hydroxyethylaminoethylamine. Other 5,8-dihydroxylated analogues can be produced in the same way using *N*-alkyl-1,2-diaminoethane derivatives. On performing reactions at or below 50°C, 1,4-bis-aminoalkylamino-anthracene-9,10-dione derivatives are formed exclusively. However, under vigorous conditions (100°C), a side reaction occurs, involving cyclization of one of the side chains to form 6-aminoalkylamino-1,2,3,4-tetrahydronaphtho(2,3-f)quinoxaline-12-dione (TNQ) (figure 3.7). It appears that anthraquinones with hydroxy-substituted chromophores are more susceptible to TNQ formation due to the hydroxy groups initiating a Michael-type cyclization reaction (Krapcho *et al*, 1991) as are compounds containing a terminal amino group which is either primary or secondary (e.g. mitoxantrone) rather than tertiary (Cheng & Zee-Cheng, 1983).

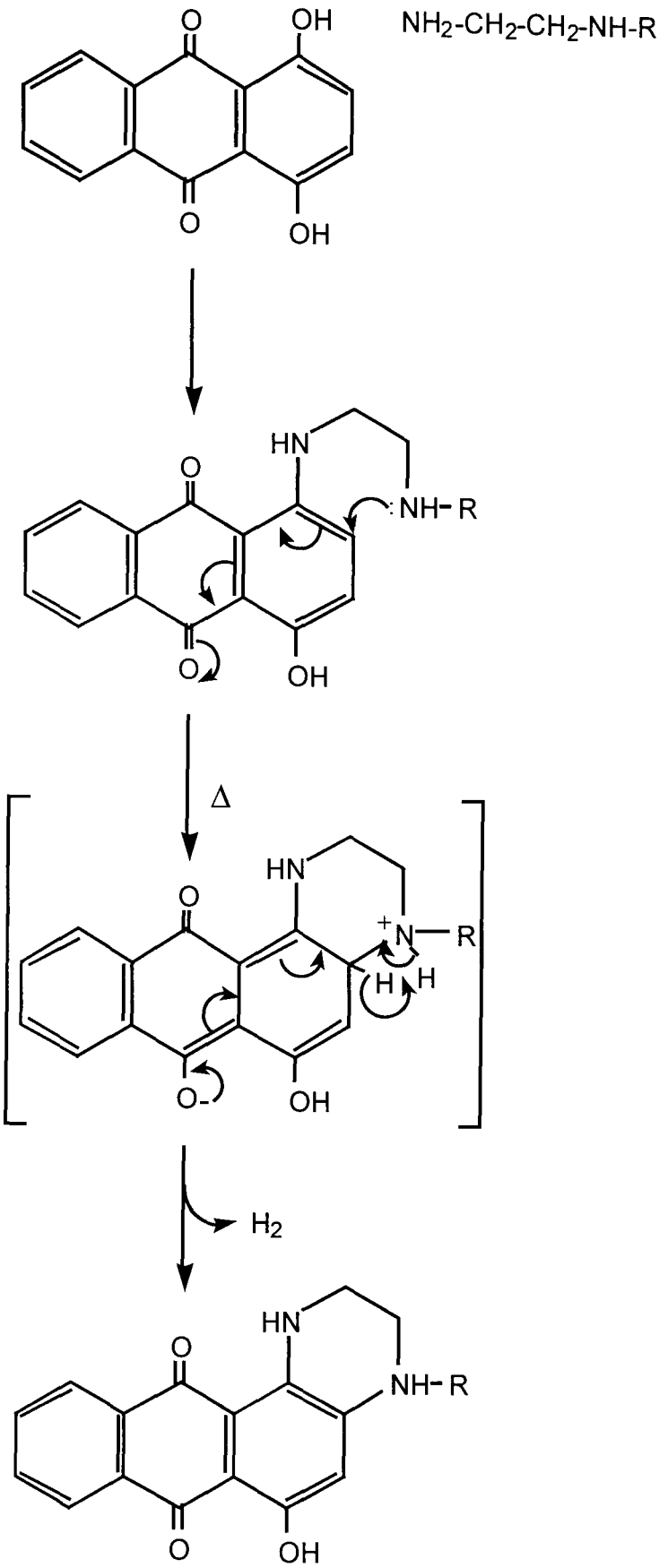
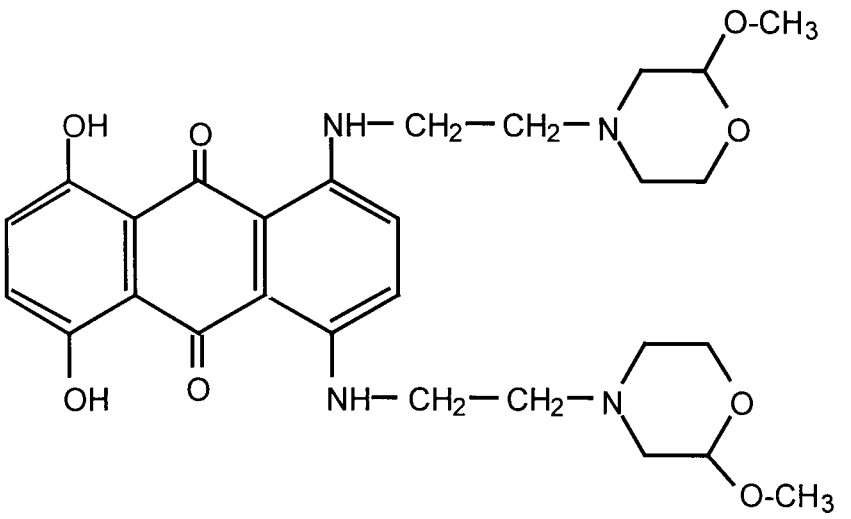
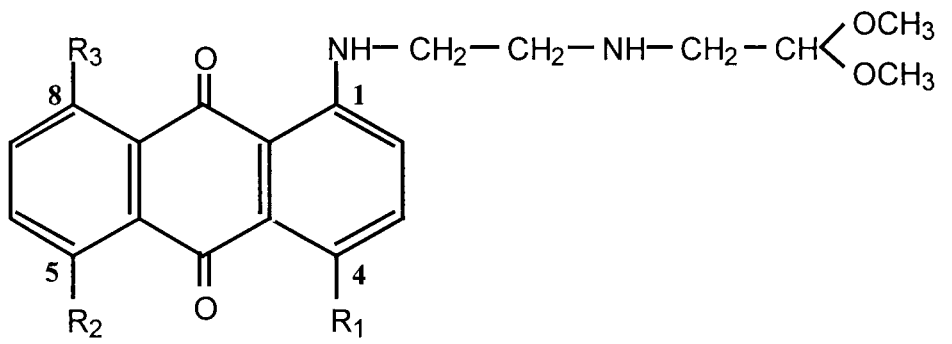


Figure 3.7: Reaction scheme showing nucleophilic substitution of leucoquinizarin with *N*-alkyl-1,2-diaminoethane derivatives, followed by cyclization to produce the tetrahydronaphthoquinoxaline (TNQ) derivative at higher temperatures.

3.3.3 SYNTHESIS OF ACETALANTHRAQUINONES



YCG4



YCG5 R_1=H, R_2=R_3=OH

YCG6 R_1=$NHCH_2CH_2NHCH_2CH(OCH_3)_2$, R_2=R_3=OH

YCG7 R_1=R_2=R_3=H

YCG8 R_1=$NHCH_2CH_2NHCH_2CH(OCH_3)_2$, R_2=R_3=H

YCG9 R_2=$NHCH_2CH_2NHCH_2CH(OCH_3)_2$, R_1=R_3=H

The amines were substituted onto the anthraquinone chromophore to give 1-, 1,4- and 1,5-substituted acetalanthraquinones. Considering the possible cyclization of the products to form substituted TNQs, all anthraquinones were synthesized at temperatures below 50°C.

Synthesis of 1-*N*-{2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamino}-anthracene-9,10-dione was achieved by nucleophilic substitution of 1-chloroanthraquinone with 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine for 9 h. The reaction was monitored by thin layer chromatography using methanol, the more polar, orange-red product having a lower Rf than the yellow starting material. On completion of the reaction, it was desirable to remove the excess amine. This would usually be achieved by vacuum distillation. As this was not possible without heating above 50°C, since it presented the risk of cyclization of the product, flash column chromatography in methanol was used and the excess amine remained on the baseline. Upon storing the fraction containing anthraquinone product at 4°C overnight, it was found that the product recrystallized from methanol. Recrystallization was therefore used to purify all subsequent preparations of the compound. The drug structure was verified by all analytical data (see section 3.2.2.1 and appendix A4).

Synthesis of 1,4-Bis-*N*-{2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamino}-anthracene-9,10-dione was carried out by nucleophilic substitution of leucoquinizarin with a large excess of 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine. Reoxidation of leucoquinizarin to quinizarin was prevented by bubbling the amine with argon for 30 min prior to addition of the anthraquinone and continuously throughout the reaction. The progression of the reaction was monitored by thin layer chromatography using a CH_2Cl_2/methanol 4:6 mixture, the characteristic 1,4 di-substituted purple-blue product being more polar than the starting material. After reacting for 8 h, the mixture was stirred vigorously in air overnight to oxidize the product. Flash column chromatography using a mixture of CH_2Cl_2 and methanol (4:6) was performed to remove the excess amine, which remained at the base line.

Attempts to recrystallize the product from various combinations of solvents including CH_2Cl_2, methanol, ethyl acetate, chloroform, acetone and butanone proved unsuccessful, and as higher alcohols could not be used in case they displaced the methoxy groups of the side chains, the product was isolated by flash column chromatography. The drug structure was verified by all analytical data (see section 3.2.2.2 and appendix A4).

Synthesis of 1,5-Bis-*N*-{2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamino}-anthracene-9,10-dione was achieved by nucleophilic substitution of 1,5-dichloroanthraquinone with a large excess of 2-[*N'*-(2,2-dimethoxyethyl)amino]-ethylamine for 9 h. The reaction was monitored by thin layer chromatography using a CH_2Cl_2/methanol 4:6 mixture, which showed the conversion of the pale yellow, relatively non-polar starting anthraquinone into a more polar pink-red product. A minor band, slightly pinker than the major product was also formed, probably the mono-substituted 5-chloro derivative. After removing the excess amine by flash column chromatography, attempts were made to recrystallize the product. However, as for the 1,4-bis substituted compound, these failed, and so again, the flash column chromatography was chosen as the method of isolation, using a mixture of CH_2Cl_2 and methanol (4:6). The drug structure was verified by all analytical data (see section 3.2.2.3 and appendix A4).

While the synthesis of the non-hydroxylated derivatives was relatively straight forward, synthesis of the 5,8-dihydroxylated compounds proved more difficult. Synthesis of 1-*N*-{2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamino}-5,8-dihydroxy-anthracene-9,10-dione was attempted by nucleophilic substitution of 2,3-dihydro-1,4,5,8-tetrahydroxyanthracene-9,10-dione with 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine, using a 2:1 molar ratio of starting materials and methanol as solvent. The relatively low proportion of amine, added slowly to the anthraquinone, and the presence of solvent should have favoured the formation of the mono-substituted compound. Reoxidation of the starting material was prevented by

performing the reaction under argon.

The reaction was initially performed at 50°C. After treatment with NaOH to re-oxidise the mixture, this yielded a major purple product (~55%), a blue product (~35%) and a plum coloured compound (~10%) which were all more polar than the brown starting material (Rf 0.99). The presence of the blue and plum coloured impurities complicated the synthesis as they were very difficult to separate from the product and the hygroscopic nature of the reaction mixture made it particularly difficult to handle. An additional difficulty was the limitation in the combinations of solvents that could be used that did not include alcohols (other than methanol). In view of this, the reaction was subsequently performed at room temperature for 4 h. However, this yielded the same products in approximately the same proportions as before. After removing as many of the impurities as possible, the mixture was analysed. The mass spectrum of the reaction mixture showed major peaks at 149 (free amine), 403 (M+1), 419 and 447. It appears from this that the plum coloured impurity was the 2-substituted compound (M+1=419). The peak at 447 may correspond to an asymmetrical 1,4-bis-substituted anthraquinone, substituted with 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine at one position and diaminoethane at the other. This was probably a component of the blue impurity seen, which may have been a mixture of several compounds.

Synthesis of 1,4-Bis-*N*-{2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamino}-5,8-dihydroxy-anthracene-9,10-dione was attempted by nucleophilic substitution of 2-[*N'*-(2,2-dimethoxyethyl)amino]ethylamine and 2,3-dihydro-1,4,5,8-tetrahydroxyanthracene-9,10-dione in a molar ratio of 20:1, the large excess of amine promoting the formation of the di-substituted compound. Synthesis was initially attempted by reacting 2,3-dihydro-1,4-5,8-tetrahydroxyanthracene-9,10-dione with the amine under argon at 50°C for 8 h.

After re-oxidation of the mixture with NaOH, and flash column chromatography using a 19:1 CH_2Cl_2/methanol mixture, several components were detected. These were a major blue product (80%) and several other blue compounds (lower Rf values than the

major product) all being more polar than the brown starting material (Rf 0.99). The similar polarity of the compounds made their separation extremely difficult. In addition, it was thought that the product may have cyclized to form a substituted TNQ and it was therefore decided to carry out the reaction at room temperature for 24 h. This yielded the major blue product and one minor blue product. At this stage, the mixture appeared stable. The major blue product was isolated by flash column chromatography. However, this resulted in the array of blue compounds previously formed. The impurities comprised approximately 5% of the total material. The product was dried and stored over P_2O_5 *in vacuo* but proved unstable, the impurities increasing to ~40-50% within 72 h. Further attempts to purify the compound, which was then stored at -15°C, still failed to achieve stability. Nevertheless, the impure mixture had characteristic visible absorbance spectrum of 1,4-bis-substituted-5,8-dihydroxy anthraquinone and its mass spectrum generated a molecular ion compatible with the required structure (M+1=533), suggesting the product was present. Other peaks were detected at 149, 279 and 391.

Degradation of YCG6 may have occurred as a result of substitution of one of the side chains onto the 2-position of the anthraquinone chromophore, to form a substituted TNQ. However, while this process is known to be enhanced by the presence of hydroxy groups at the 5 and 8 positions of the anthraquinone ring, which initiate a Michael type reaction (see section 3.3.2), it usually occurs at temperatures above 50°C, and the array of impurities formed at 50°C were also seen when the reaction was carried out at room temperature. Furthermore, in all probability, the cyclized product would be of a different polarity and colour to the non-cyclized drug (Blanz *et al*, 1991a; Mewes *et al*, 1993) and thus would be easily distinguishable by thin layer chromatography. Alternatively, the drug may have polymerized, via reaction of the side chains, either inter- or intra-molecularly. However, again, such products would be markedly different from YCG4 and thus clearly visible by thin layer chromatography. Finally, degradation may have resulted from hydrolysis of the acetal groups or cyclization of the side chain(s) although neither of these occurred in the synthesis of the non-hydroxylated analogues.

Furthermore, cyclization is less likely to occur once the amine is attached to the anthraquinone ring as the lone pair of electrons on the amine nitrogen become more delocalized. Thus, side chain hydrolysis appears to be the only feasible explanation. This is supported by the multiple components of similar nature revealed by thin layer chromatography, which suggest a process in which several compounds analogous to the acetalanthraquinone were generated (see section 4.3.2). To test this hypothesis, the compound was hydrolysed in 0.1M HCl for 10 min at room temperature. This produced an increase in all three impurities, supporting degradation of the acetal groups. It appears that hydrolysis was promoted by the silica used during flash column chromatography, as the compound was stable until this was carried out.

Crude *N*-[2-(2′-methoxymorpholinyl)ethyl]amine was reacted with 2,3-dihydro-1,4,5,8-tetrahydroxyanthracene-9,10-dione in an attempt to produce 1,4-Bis-*N*-{[2-(2'-methoxymorpholinyl)ethyl]amino}-5,8-dihydroxy-anthracene-9,10-dione. Synthesis was achieved by heating an excess of the amine mixture with the anthraquinone under argon for 12 h. The reaction, monitored by thin layer chromatography using a 99:1 CH_2Cl_2/TEA mixture, revealed one major blue compound (Rf 0.72) which was more polar than the brown starting material (Rf 0.99) and several colourless impurities, detected using iodine and ninhydrin. The major blue compound, assumed to be the required product, was collected by flash column chromatography using a mixture of CH_2Cl_2 and TEA (99:1). After removing silica and solvents and drying *in vacuo* over P_2O_5, analysis of the material by thin layer chromatography revealed several of the colourless impurities were still present, and these subsequently proved impossible to separate from the anthraquinone product. One reason for the difficulty in removing these impurities was that there were several of them, each with different chemical properties. Another reason was the potential instability of the morpholino ring in acidic environments, which excluded the use of many reagents for separation/purification. The low yield (~2%) prevented full analysis of the compound but analysis by mass spectrometry indicated peaks at 130, 144 and 556 (M+1) (see appendix A4). The peaks

at 130 and 144 may have corresponded to either unreacted morpholino alcohol or fragments of amine. In addition, the mixture had characteristic visible absorbance spectrum of 1,4-bis-substituted-5,8-dihydroxy anthraquinone, with a λmax of 674nm (see section 2.3.2.2). The compound appeared to be of a relatively lipophilic nature compared to other 1,4-bis-substituted anthraquinones. This is consistent with the more lipophilic nature of morpholino doxorubicin and methoxymorpholino doxorubicin compared with the parent compound doxorubicin (Acton *et al*, 1984; Facchetti *et al*, 1991; Ripamonti *et al*, 1992).

As the 5,8-dihydroxylated compounds could not be isolated in a pure form, only the non-hydroxylated analogues (YCG7, YCG8 and YCG9) were used to investigate the potential conversion of acetal-containing anthraquinones to their corresponding aldehyde derivatives.

CHAPTER 4: CHEMICAL AND METABOLIC ACTIVATION OF ACETALANTHRAQUINONES

4.1 INTRODUCTION

Having synthesized the acetalanthraquinones, the purpose of this study was to investigate possible chemical and metabolic routes that could be used to yield their respective aldehyde derivatives. With this objective in mind, acid-catalysed hydrolysis of the acetalanthraquinones was studied at various temperatures and the physico-chemical parameters of the reaction were then determined. Oxidative metabolism by NADPH-fortified mouse liver microsomes *in vitro* was also investigated.

4.2 METHODS AND MATERIALS

Acetalanthraquinones were synthesized as described in sections 3.2.2.1-3.2.2.3. Balb C mouse liver microsomes from 6-8 week old mice, in 100mM Tris-HCl buffer, pH 7.4, were supplied by the Cancer Research Campaign, Beatson Labs, University of Glasgow, UK. Details of the spectral determination of the cytochrome P-450 content of these microsomes can be found in appendix A5. Solvents were supplied by Fisons, Loughborough, UK and were HPLC grade. All other reagents were supplied by Sigma Chemical Co., Poole, Dorset, UK and Aldrich Chemical Co., Gillingham, Dorset, UK, unless otherwise stated and were 98-99% pure. DMSO was spectrophotometric grade. The activity of NADPH was verified by absorbance measurement at 260nm and 340nm at pH10. A260:A340 = 2.27:1, in exact accordance with the value obtained by Sigma Chemical Co. Phosphate buffer for the HPLC mobile phase consisted of a mixture of Na_2HPO_4 (0.1M) and KH_2PO_4 (0.1M) pH 7.4. Where abbreviations have been used for reagents, full names can be found in appendix A8.

4.2.1 SEPARATION OF ACETALANTHRAQUINONES AND VALIDATION OF THE HPLC SYSTEM

Solutions of acetalanthraquinone ranging in concentration from 100μM -1mM in 0.1M HCl were neutralized with an equal volume of 0.1M NaOH and 50μl samples of the resulting mixture analysed by HPLC. The HPLC system consisted of a Walters 501 millipore pump, Walters 484 tunable absorbance detector and Spectra Physics data jet integrator, fitted with a reversed phase, C18, 10μm nucleosil column (250mm length x 4.6mm internal diameter). Samples were injected using a valve mechanism manual injection port (reodine) fitted with a 20μl capacity loop. The flow rate of the mobile phase (0.1M phosphate buffer, pH 7.4/ CH_3CN 1:1 v/v) was 2ml/min. This was degassed by sonication for 30 min before use.

Chromatograms were monitored at both the UV λmax and visible λmax of the drugs (254 and 499nm for YCG7, 260 and 627nm for YCG8 and 232 and 519nm for YCG9). The peak areas obtained were then plotted against drug concentration to ensure linearity. The average peak area at each drug concentration was also determined and the mean ± sd calculated to ensure consistency of the HPLC method.

4.2.2 CHEMICAL HYDROLYSIS OF ACETALANTHRAQUINONES

Solutions of drug (ranging in concentration from 100μM-1mM) in 0.1M HCl, were heated to 90°C in sealed Eppendorf tubes for 0-360 min. Drug in double distilled water at pH 7.4, heated to 90°C for 360 min, and drug in 0.1M HCl, maintained at 37°C for 360 min, were also set up as controls. At intervals of 15 min, 50μl of each test solution was removed, neutralized with 50μl of acetone-ice-cold NaOH (0.1M) and analysed by HPLC, as described in section 4.2.1. The procedure was repeated at temperatures of 70°C and 80°C, monitoring the samples at regular intervals. The disappearance of the parent peak over the range of temperatures and drug concentrations was used to calculate the rate of hydrolysis and activation energy of the reaction for each compound.

4.2.3 OXIDATIVE METABOLISM OF ACETALANTHRAQUINONES

A homogeneous suspension of Balb C mouse liver microsomes (final concentration 1mgml^{-1}) was added to Erlermeyer flasks containing acetalanthraquinone (1mM) and NADPH (0.1M in phosphate buffer, pH 7.4) such that the total volume was 500µl (control flasks contained phosphate buffer instead of NADPH). The solutions were incubated in a shaking water bath at 37°C for 60 min. Following this, 200µl of each incubate was removed and added to an equal volume of acetone-ice-cold acetonitrile to terminate the reaction. The resulting mixture was centrifuged at 14 000 x g for 15 min (A14 microcentrifuge, Jouan Ltd., Milan, Italy) to spin down microsomal protein, and the supernatant analysed by HPLC, as described in section 4.2.1. The rate of metabolism of each acetalanthraquinone (nmolmg^{-1}min^{-1}) was determined from the percentage loss of the parent compound. The effect of boiled microsomes on the acetalanthraquinones in the presence and absence of NADPH was also investigated.

4.3 RESULTS

4.3.1 VALIDATION OF THE HPLC SYSTEM

The HPLC system was found to be suitable for all drugs used. The relationship between peak area (measured at the λmax of the drug in the UV region) and drug concentration (100µM-1mM) was found to be linear (figures 4.1 to 4.3). The average peak area for each drug concentration was also determined and the standard deviations were found to be between 2.7 and 6.1% for YCG7, 4.7 and 14.3% for YCG8 and 3.1 and 4.3% for YCG9.

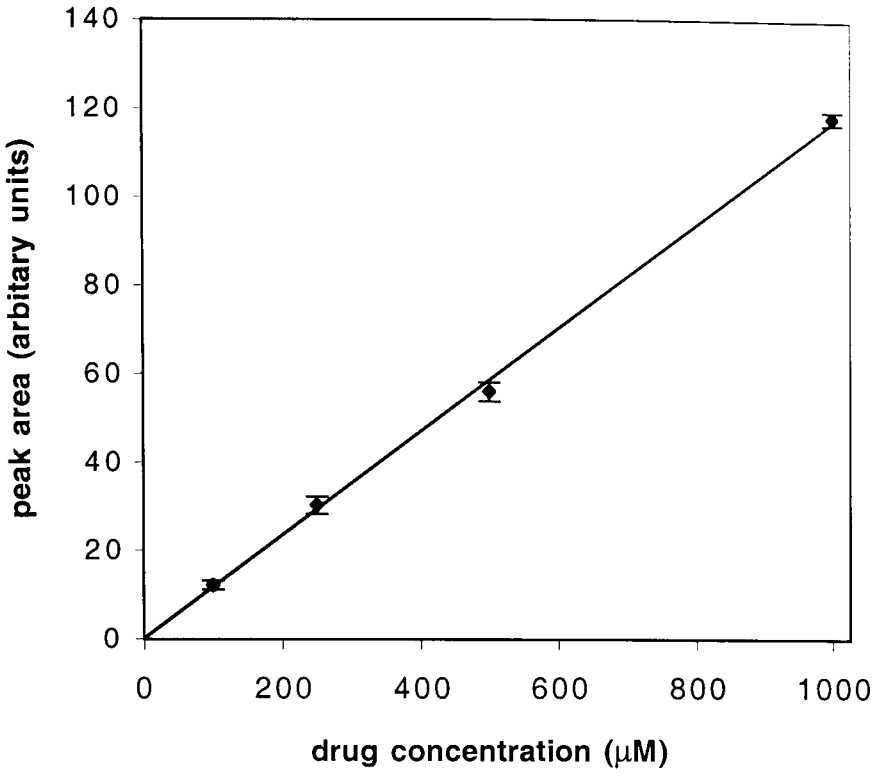


Figure 4.1: Relationship between peak area and drug concentration for YCG7. Values shown represent the mean ± sd of three separate experiments.

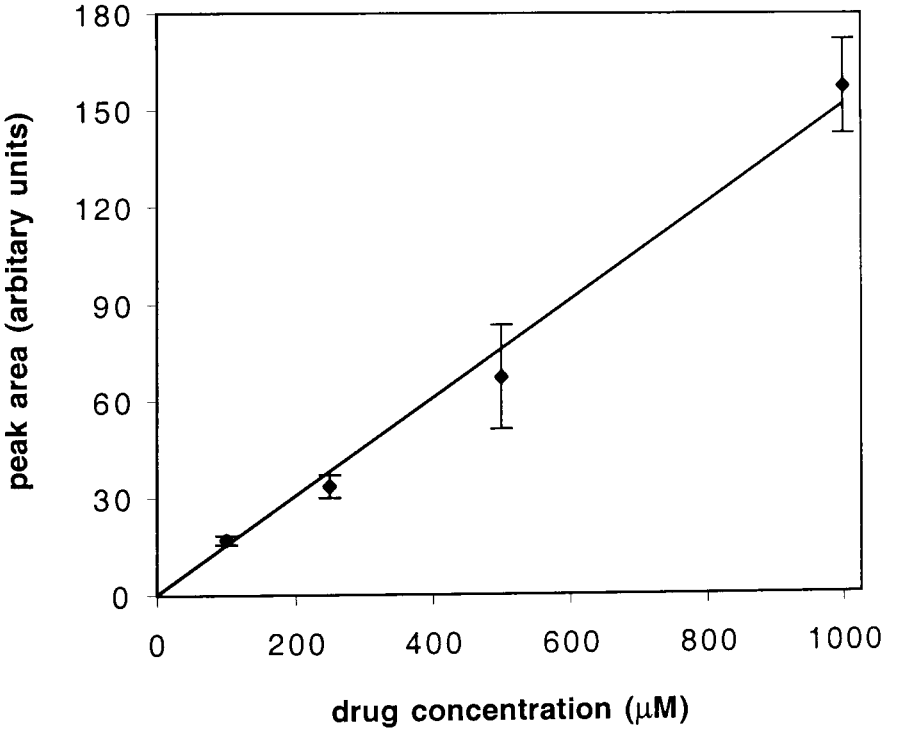


Figure 4.2: Relationship between peak area and drug concentration for YCG8. Values shown represent the mean ± sd of three separate experiments.

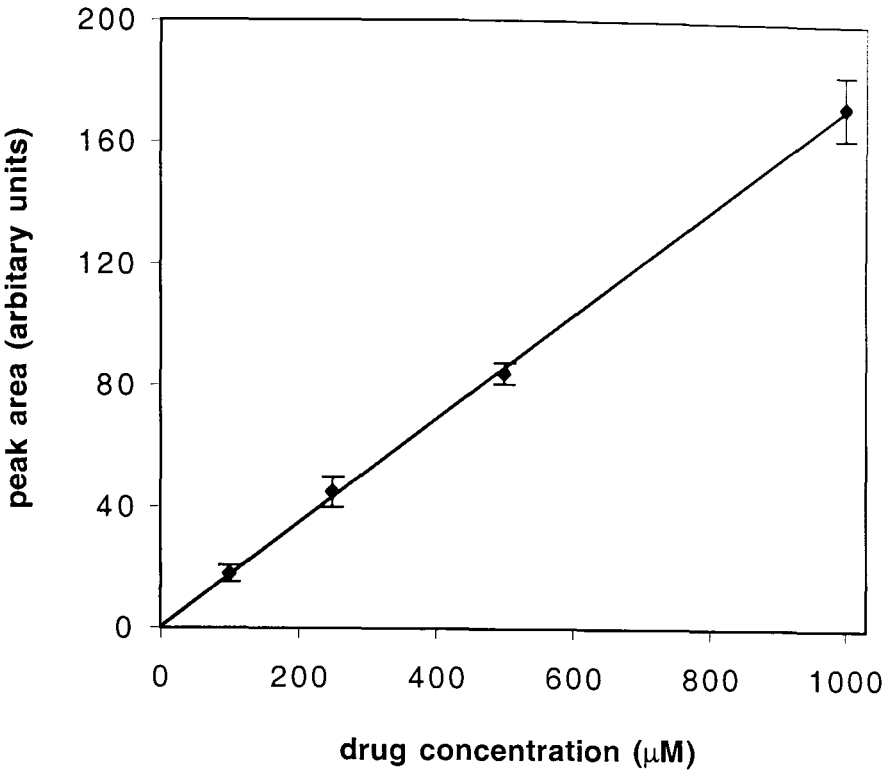


Figure 4.3: Relationship between peak area and drug concentration for YCG9. Values shown represent the mean ± sd of three separate experiments.

4.3.2 CHEMICAL HYDROLYSIS OF THE ACETAL-ANTHRAQUINONES

The retention times for ametantrone, YCG7, YCG8 and YCG9 were 3.05, 8.40, 7.63, and 6.56 min respectively (figures 4.4A to 4.6A). Due to its low solubility in water and acid, it was necessary to dissolve YCG7 in a small amount of DMSO (1% final concentration) before dissolving in aqueous solvents. This produced a peak at 1.44 min. Acid-catalysed hydrolysis of YCG7 in 0.1M HCl between 70 and 90°C produced 2 peaks in addition to the acetalanthraquinone, with retention times of 4.78 and 2.36 min (figures 4.4B and 4.4C). Hydrolysis of YCG8 produced 3 peaks in addition to the acetalanthraquinone, with retention times of 5.21, 3.00 and 2.03 min (figures 4.5B and 4.5C). Hydrolysis of YCG9 also produced 3 peaks in addition to the acetalanthraquinone, with retention times of 3.37, 2.06 and 1.24 min (figures 4.6B and 4.6C). The formation of all products increased with time.

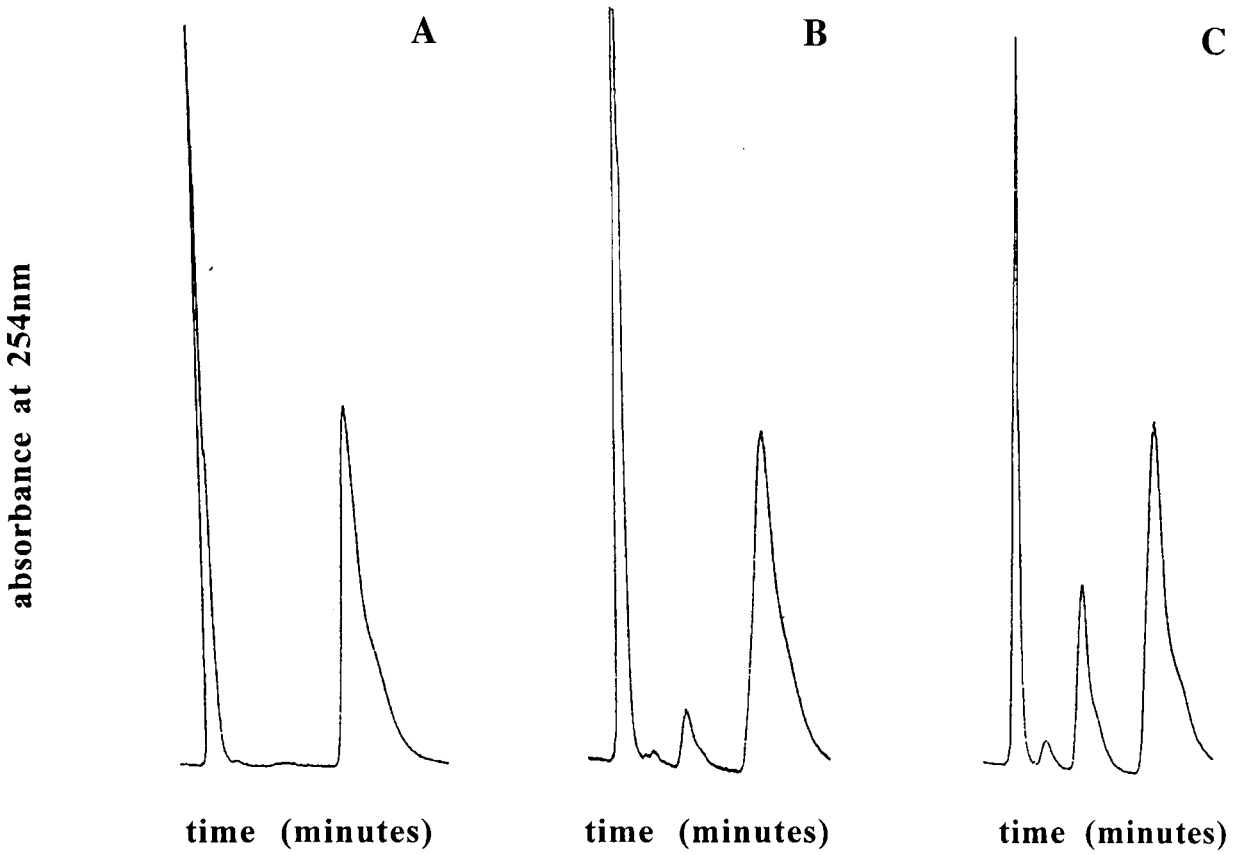


Figure 4.4: HPLC chromatograms for YCG7 after (A) 0 minutes, (B) 45 minutes and (C) 90 minutes incubation with 0.1M HCl at 90°C.

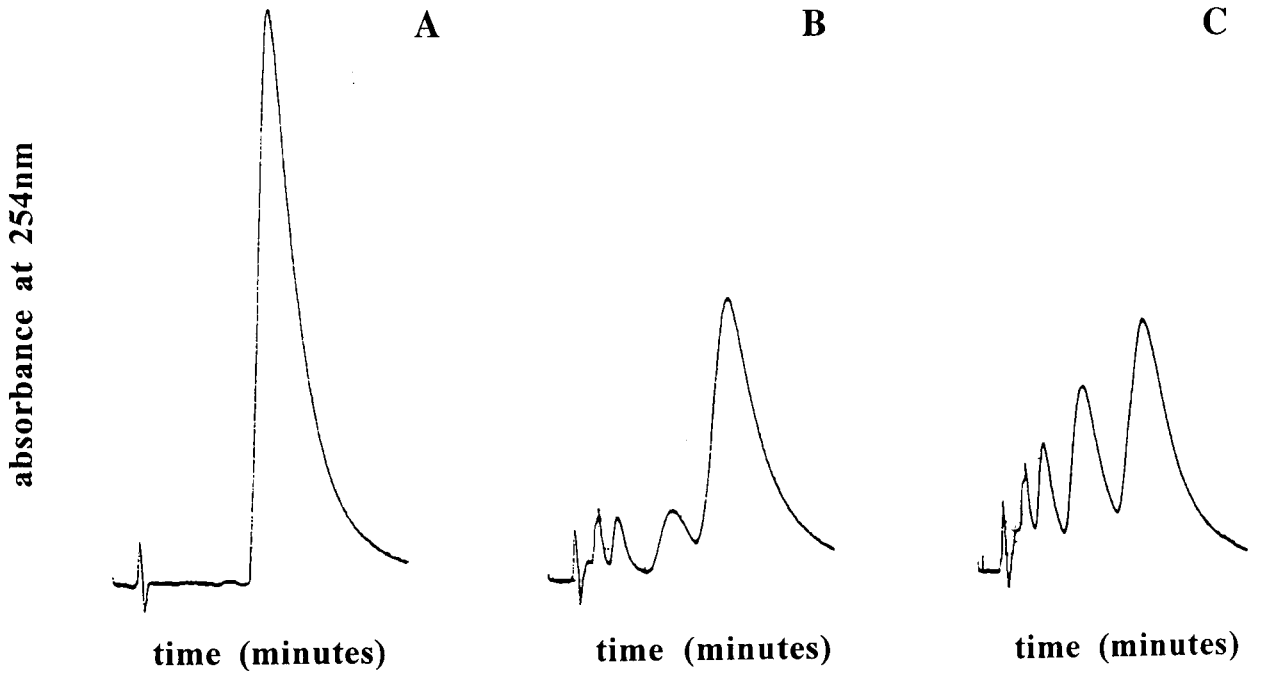


Figure 4.5: HPLC chromatograms for YCG8 after (A) 0 minutes, (B) 45 minutes and (C) 90 minutes incubation with 0.1M HCl at 90°C.

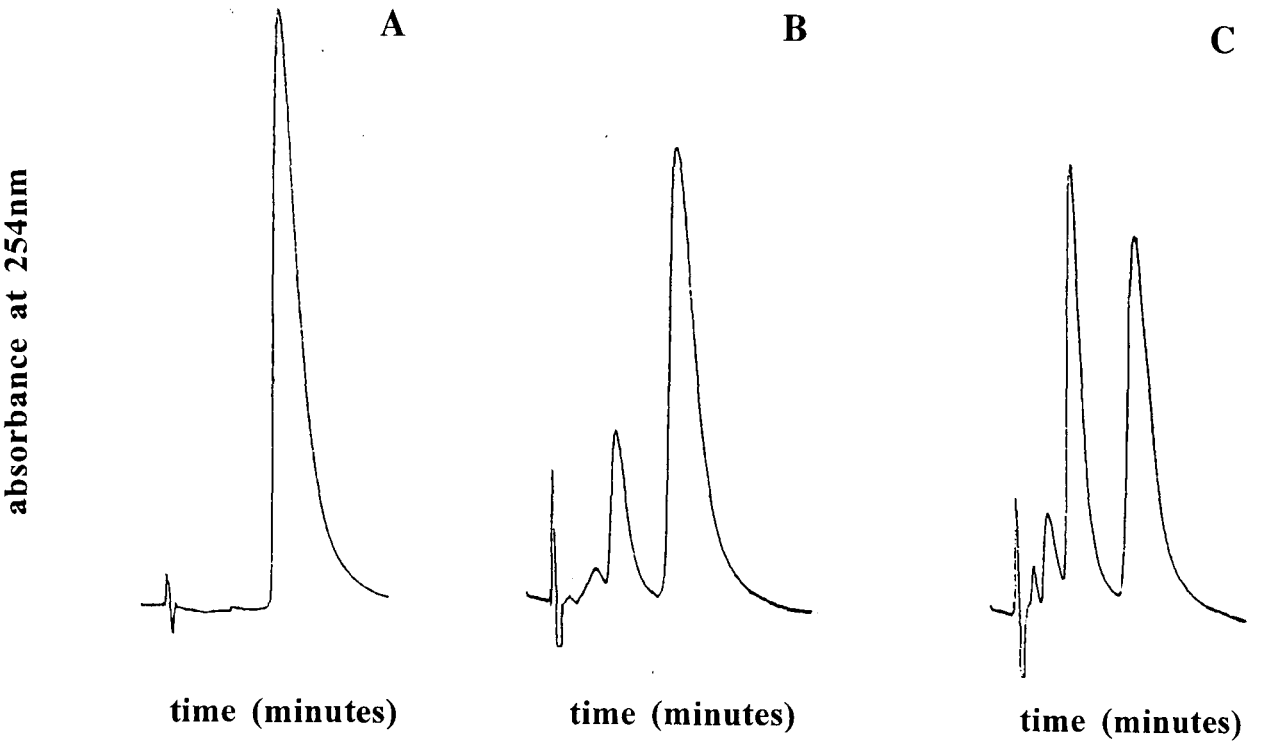


Figure 4.6: HPLC chromatograms for YCG9 after (A) 0 minutes, (B) 45 minutes and (C) 90 minutes incubation with 0.1M HCl at 90°C.

In each case, detection was carried out at the UV λmax of the starting compound, in order to detect the formation of any colourless products (none were produced). While the absorbance spectra of the hydrolysed reaction mixtures and acetalanthraquinones were identical in the visible region of the spectrum, there was a noticeable difference in the UV region, particularly for YCG8, which showed a shift in λmax from 260.3 to 250.1nm and a 3.3-fold increase in absorbance at that wavelength (figure 4.7)

Ametantrone was refractory to hydrolysis under the above conditions, the parent peak remaining intact after a 3 h incubation period (results not shown). Ametantrone incubated at 37°C with 0.1M HCl for 3 h or heated to 90°C in water for 3 h was also stable. Similarly, acetalanthraquinones incubated at 37°C with 0.1M HCl for 6 h or heated to 90°C in water for 6 h remained intact. Hydrolysis was absent at 37°C even with increased concentrations of acid (up to 1M).

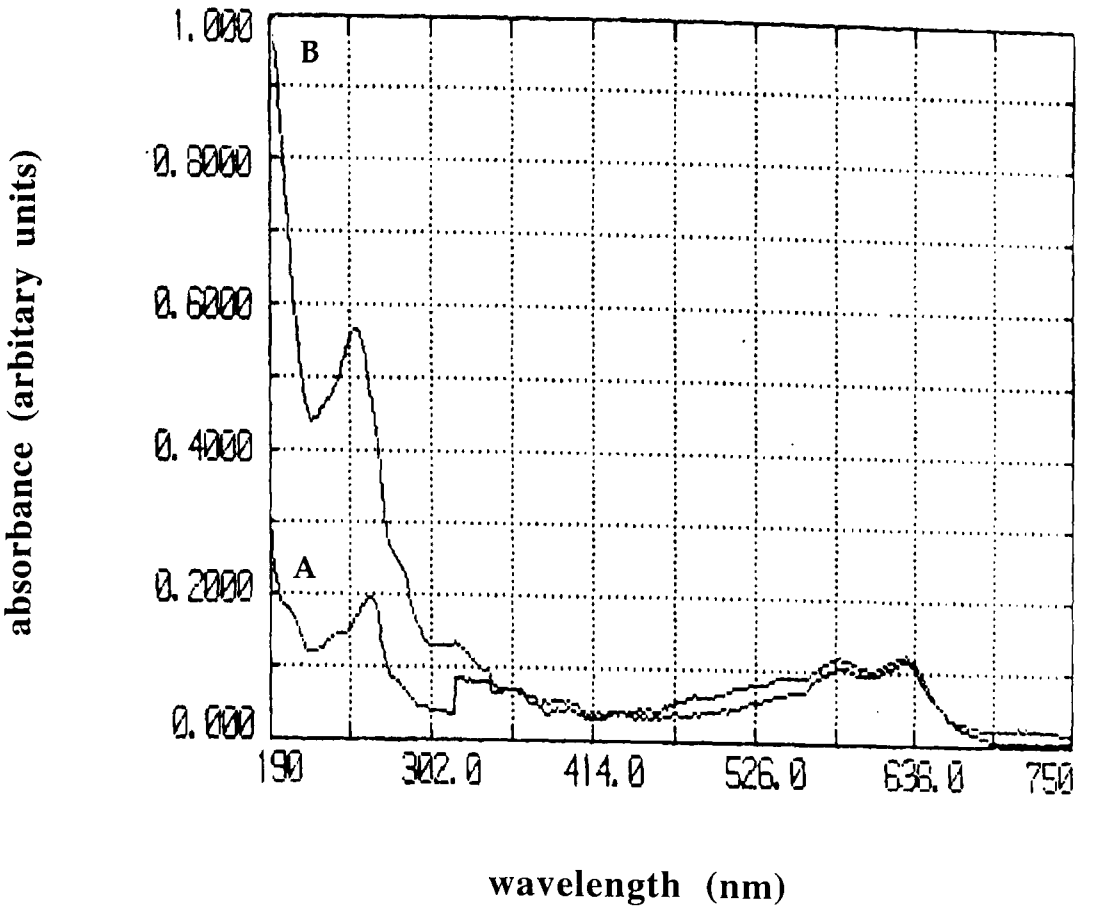


Figure 4.7: UV-visible absorption spectrum for YCG8 (A) before and (B) after 4 hours incubation with 0.1M HCl at 90°C.

4.3.3 PHYSICO-CHEMICAL PARAMETERS OF THE HYDROLYSIS REACTION

The rate constants for the hydrolysis of the acetalanthraquinones at 70-90°C are shown in table 4.1. Hydrolysis rates were calculated from percentage loss of the parent compound at its λmax value in the UV region of the spectrum. Expression of the hydrolysis rate in terms of loss of the acetalanthraquinone meant that the slight differences in λmax value between the parent compounds and the products did not interfere with the calculation.

Table 4.1: Rate constants and activation energies for the acetalanthraquinones

DRUG	k 70°C	k 80°C	k 90°C	E ($KJmol^{-1}$)
YCG7	3.5 ± 0.3	11.3 ± 0.9	34.0 ± 3.4	105.8 ± 7.4
YCG8	5.0 ± 0.4	25.7 ± 3.4	56.0 ± 9.7	117.9 ± 10.4
YCG9	8.8 ± 1.4	28.5 ± 5.7	75.5 ± 8.6	104.2 ± 6.2

All compounds were hydrolysed in 0.1M HCl. The rate constant (k) at each temperature was calculated from a plot of log a/a-x vs. t and the values shown represent k $x10^4$ (min^{-1}). Activation energies (E) were calculated from a plot of log k vs 1/T (see figures 4.8, 4.10 and 4.12). Values represent the mean ± sd for three separate experiments performed in triplicate.

The hydrolysis reaction was found to be temperature-dependent and followed *pseudo* first order kinetics (see appendix A6 for details).

For a first order reaction:

$$\log [a/(a-x)] = kt/2.303$$

where *a* is the initial concentration of the reactant (drug) and *x* is the concentration of reactant utilized during time *t* of the reaction (Morris, 1985). A straight line plot of log a/(a-x) vs t was made in order to calculate the rate constant, k, by linear regression, where k = gradient of the line (m) x 2.303 (figures 4.8, 4.10 and 4.12). The apparent rates of hydrolysis were YCG9>YCG8>YCG7. However, the third product for YCG8 appeared to form more quickly than the corresponding product for YCG9 (5.1% ± 0.3 in 90 min for YCG8 compared to 2.6% ± 0.4 for YCG9).

The activation energies for the hydrolysis of YCG7, YCG8 and YCG9 were calculated from the Arrhenius equation (Morris, 1985) as follows

$$\log k = \log A - E/2.303R \times 1/T$$

where k is the rate constant, R is the gas constant, T is the temperature (in Kelvin) and A is the Arrhenius constant. The activation energy can be calculated by plotting log k vs 1/T (figures 4.9, 4.11 and 4.13). The slope of this line equals -E/2.303R; as 2.303R equals $19.14 JK^{-1}mol^{-1}$, the value of the activation energy can be determined from the Arrhenius plot as being equal to -(gradient of the line x 19.14) $Jmol^{-1}$. The activation energies were found all to be in the same order: 105.8 ± 7.4, 117.9 ± 10.4 and 104.2 ± 6.2 $KJmol^{-1}$ for YCG7, YCG8 and YCG9 respectively.

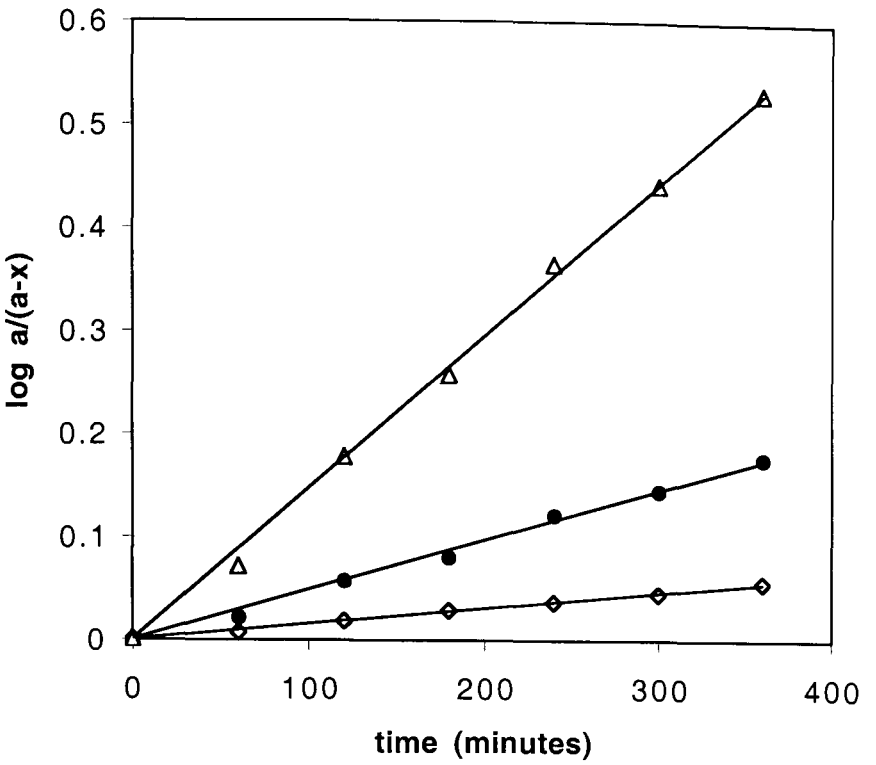


Figure 4.8: Rate constants (k) for the hydrolysis of YCG7 at 70°C (◊), 80°C (•) and 90°C (Δ). Values shown represent the mean ± sd of three separate experiments.

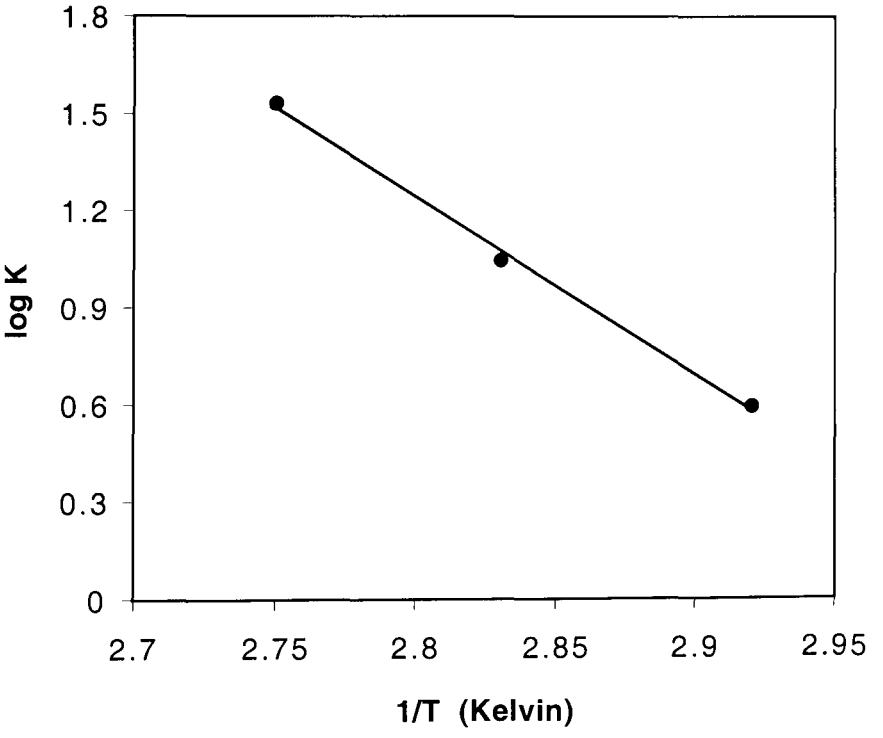


Figure 4.9: Activation energy for the hydrolysis of YCG7, calculated from the rate constants at 70°C, 80°C and 90°C. Values shown represent the mean ± sd of three separate experiments.

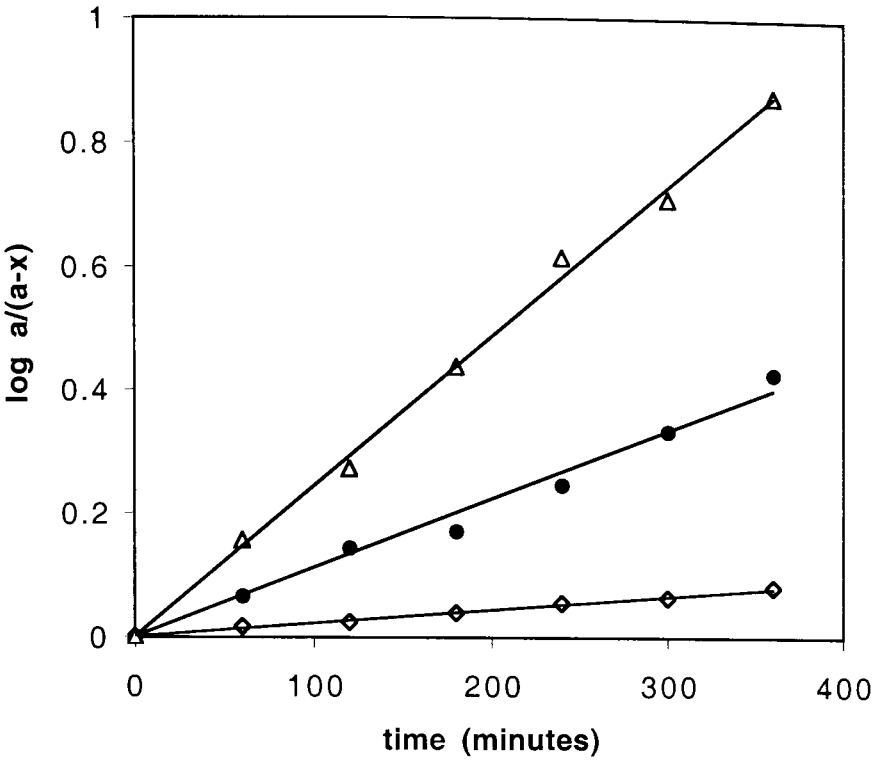


Figure 4.10: Rate constants (k) for the hydrolysis of YCG8 at 70°C (◊), 80°C (•) and 90°C (Δ). Values shown represent the mean ± sd of three separate experiments.

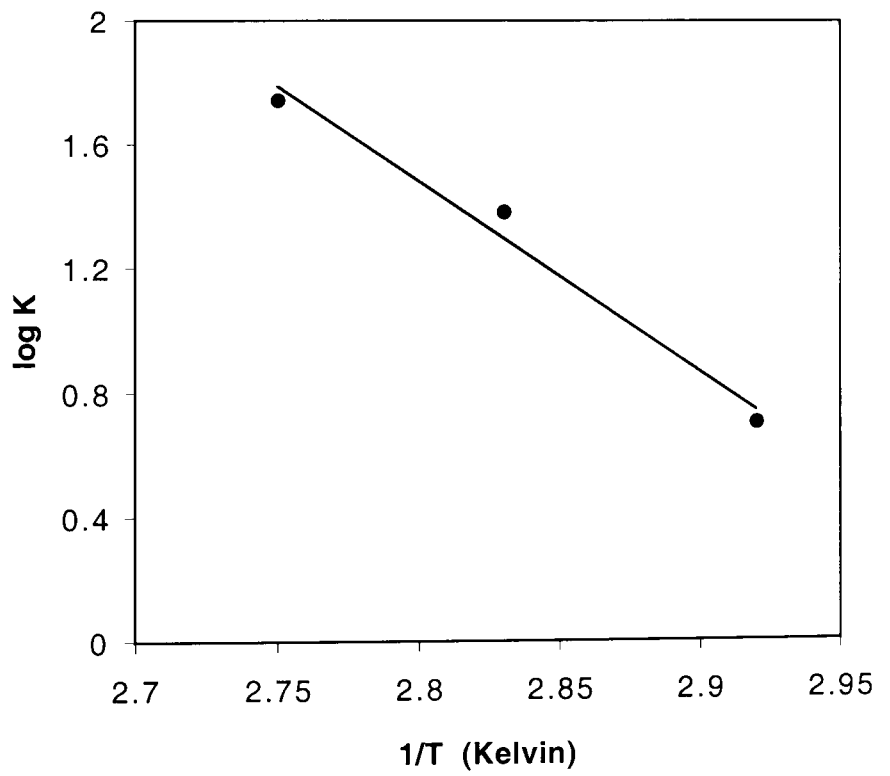


Figure 4.11: Activation energy for the hydrolysis of YCG8, calculated from the rate constants at 70°C, 80°C and 90°C. Values shown represent the mean ± sd of three separate experiments.

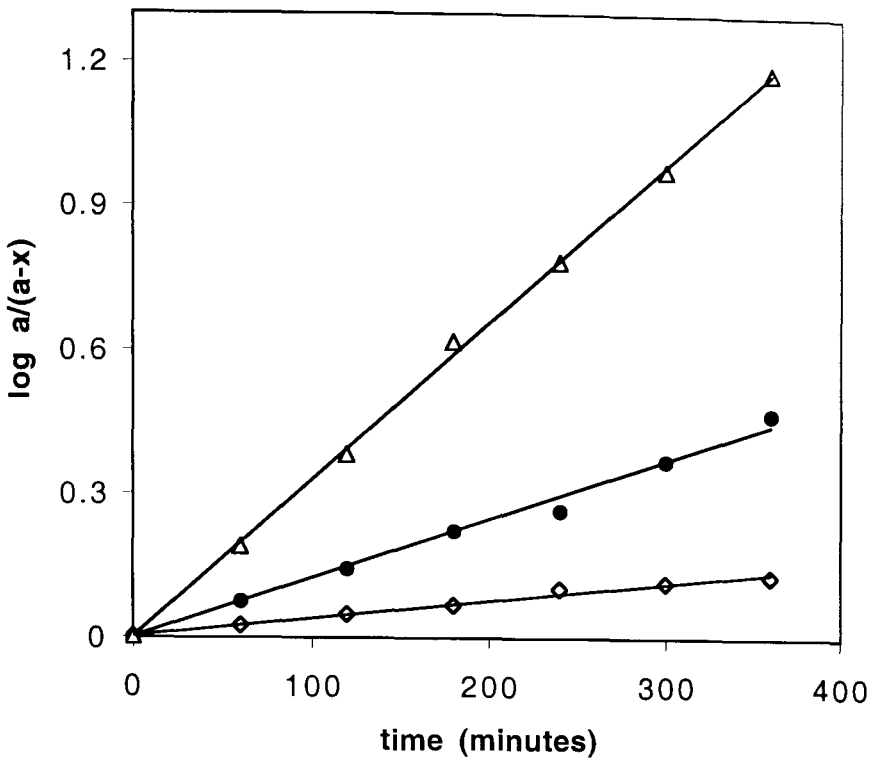


Figure 4.12: Rate constants (k) for the hydrolysis of YCG9 at 70°C (◊), 80°C (•) and 90°C (Δ). Values shown represent the mean ± of three separate experiments.

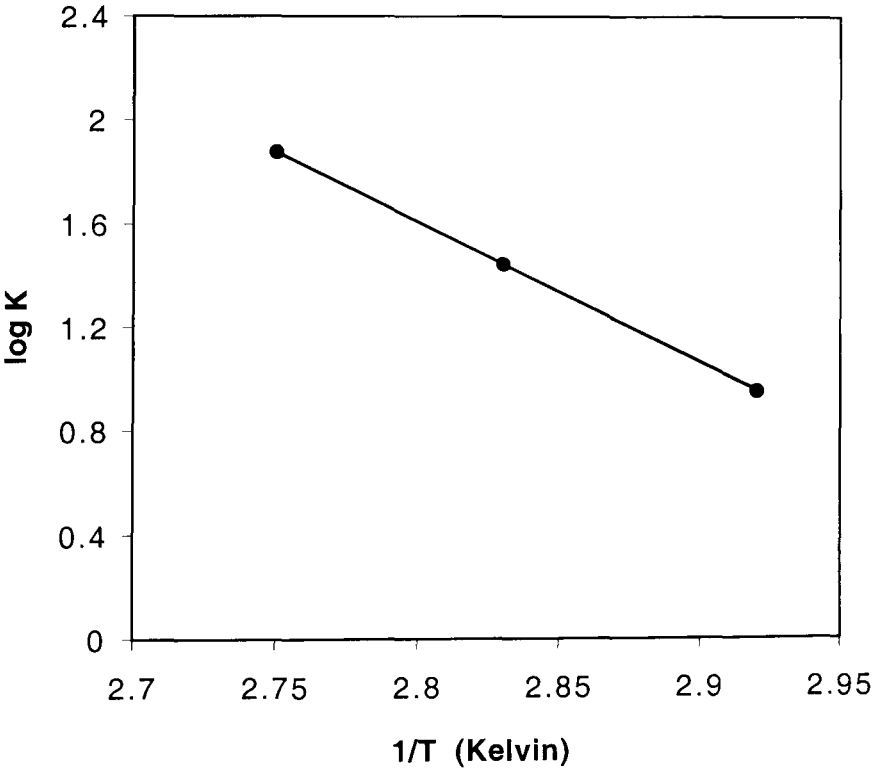


Figure 4.13: Activation energy for the hydrolysis of YCG9, calculated from the rate constants at 70 °C, 80 °C and 90° C. Values shown represent the mean ± sd of three separate experiments.

4.3.4 OXIDATIVE METABOLISM OF THE ACETALANTHRAQUINONES

Incubation of YCG7 with Balb C male mouse liver microsomes at 37°C for 60 min in the presence of NADPH produced 2 peaks, with retention times of 7.94 and 3.21 min, in addition to the acetalanthraquinone which had a retention time of 10.77 min (figure 4.14). Incubation of YCG8 under the same conditions resulted in 3 peaks with retention times of 6.84, 4.14 and 2.70 min, in addition to the acetalanthraquinone, which had a retention time of 10.08 min (figure 4.15). Incubation of YCG9 also resulted in 3 peaks with retention times of 6.13, 4.12 and 3.55 min, in addition to the acetalanthraquinone, which had a retention time of 9.26 min (figure 4.16). All products were detected in both the UV and visible regions of the spectrum. The visible absorbance spectra of the metabolite mixtures were identical to those of the acetalanthraquinones, demonstrating that the chromophore remained intact following metabolism (results not shown). No metabolism was detected when the drugs were incubated in the absence of NADPH or when using boiled microsomes. Following centrifugation of the microsome-drug mixtures containing NADPH, the microsomal pellets were seen to be coloured, indicating the binding of drug to the microsomal protein. A small amount of this drug remained bound to the microsomes even on the addition of acetonitrile. This was not observed in the absence of NADPH, suggesting the metabolites generated possessed an increased affinity for microsomal protein. For this reason, the percentage metabolism was calculated from the loss of parent compound, rather than product formed. The values obtained following a 60 min incubation period were $7.2 \pm 0.6 nmol^{-1}mg^{-1}min^{-1}$ (43.2% ± 3.9) for YCG7, $9.0 \pm 0.05 nmol^{-1}mg^{-1}min^{-1}$ (54.0% ± 0.3) for YCG8 and $9.1 \pm 0.2 nmol^{-1}mg^{-1}min^{-1}$ (54.7% ± 0.9) for YCG9.

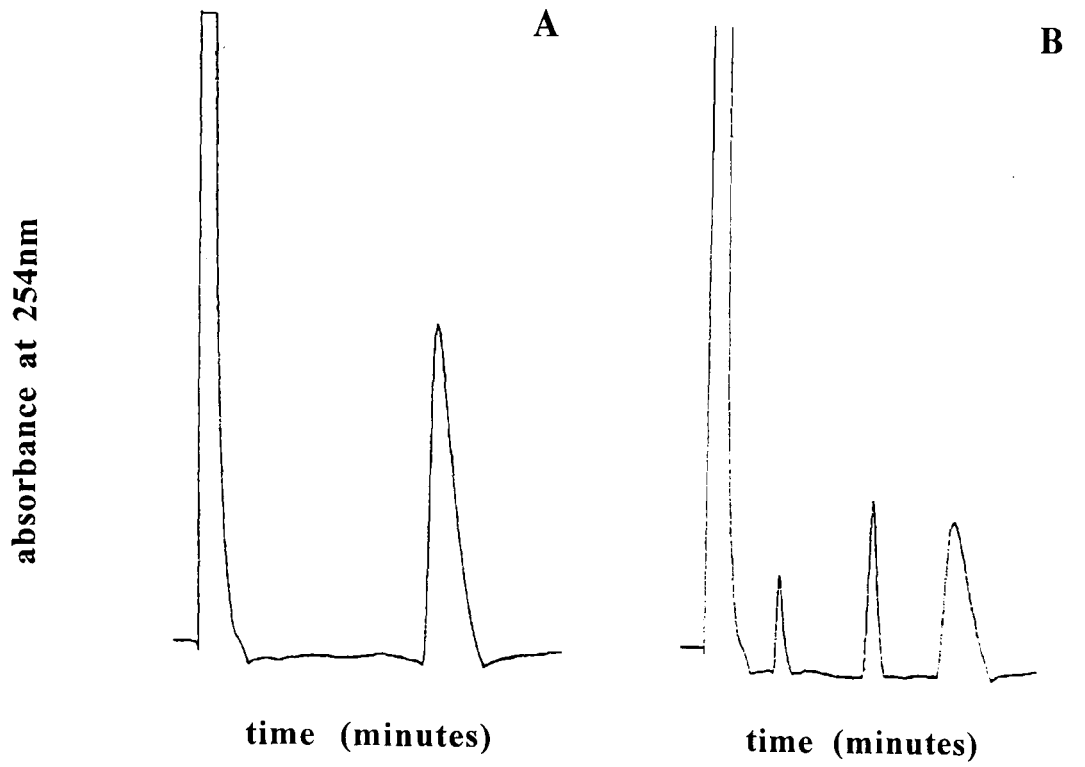


Figure 4.14: HPLC chromatograms for YCG7 after 60 minutes incubation with Balb C mouse liver microsomes (A) -NADPH and (B) +NADPH.

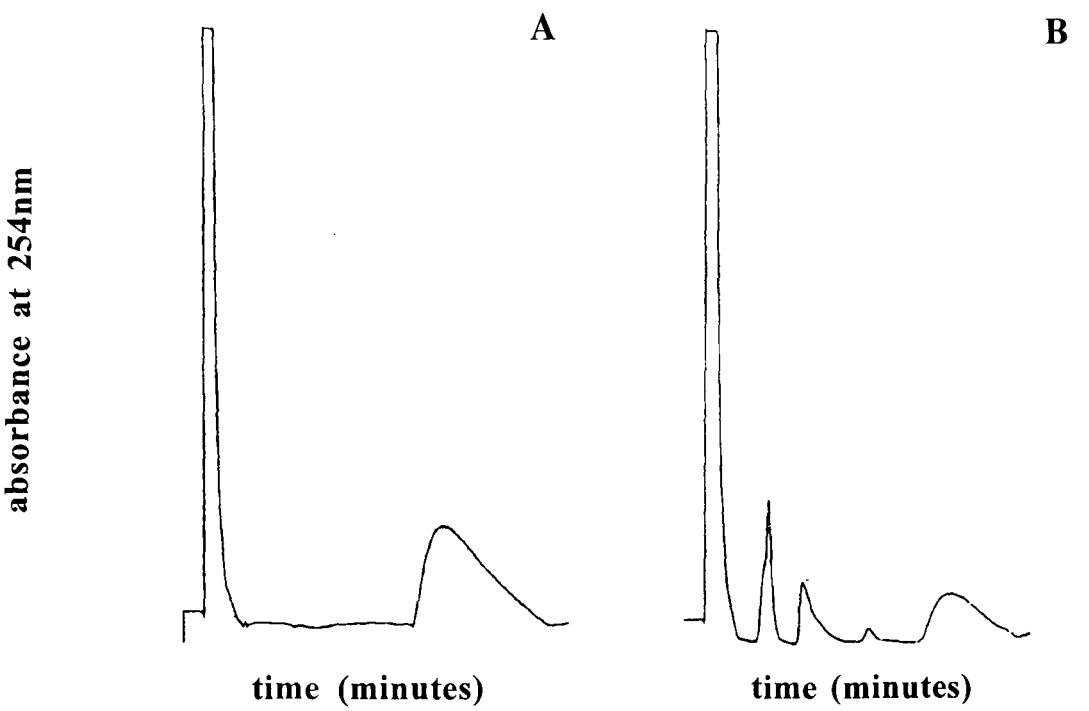


Figure 4.15: HPLC chromatograms for YCG8 after 60 minutes incubation with Balb C mouse liver microsomes (A) -NADPH and (B) +NADPH.

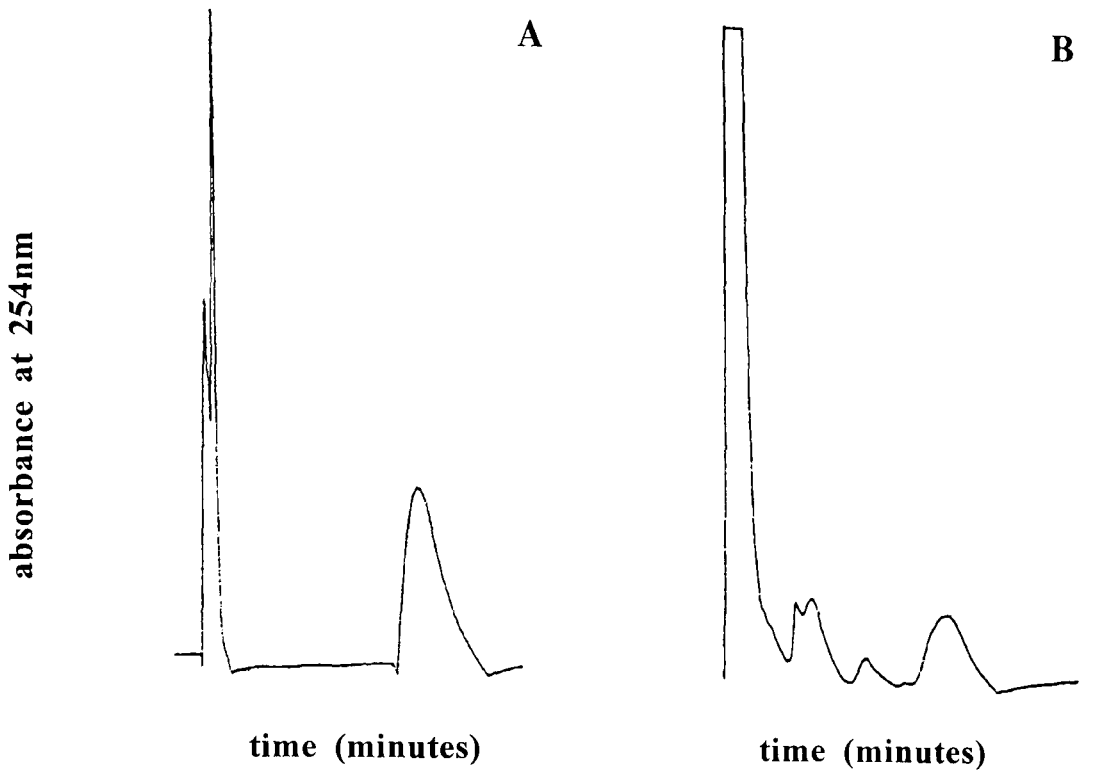


Figure 4.16: HPLC chromatograms for YCG9 after 60 minutes incubation with Balb C mouse liver microsomes (A) -NADPH and (B) +NADPH.

4.4 DISCUSSION

4.4.1 CHEMICAL HYDROLYSIS OF THE ACETALANTHRAQUINONES

While acetals are stable in non-acidic solution (Fessenden & Fessenden, 1986), they are easily cleaved by dilute acids. Hydrolysis of acetals occurs due to the electron withdrawing effect of the methoxy groups, rendering the C atom to which they are attached δ^+ and therefore susceptible to attack by nucleophiles such as water, under acidic conditions. This is enhanced by the electron donating effect of the methyl portion of the methoxy group, which encourages protonation of the oxygen atom, resulting in elimination of the *O*-methyl group in the form of methanol to generate a hemi-acetal. Elimination of another molecule of methanol by the same mechanism generates the geminal diol (1,1-diol), which breaks down spontaneously to produce the corresponding aldehyde. Although the reaction is reversible, in the equilibrium between an aldehyde.

hemiacetal, & acetal, the aldehyde is generally favoured (Fessenden & Fessenden, 1986). In the presence of oxygen and UV light, the aldehyde product can be further oxidized to produce the corresponding carboxylic acid.

Hydrolysis of acetals usually occurs by the S_N1 mechanism, a non-concerted process in which protonation of the acetal is followed by rate-limiting breakdown of the protonated substrate to an alcohol and a resonance stabilized carbocation (Fife, 1972) (figure 4.17). However, in some cases, there is evidence that a bimolecular (S_N2) mechanism operates, in which preequilibrium protonation of the acetal is followed by rate determining attack of water on the protonated acetal, with concerted cleavage of the C-O bond (Elaison & Kreevoy, 1978) (figure 4.18). As S_N2 reactions are strictly dependent on steric accessibility, this mechanism is often unfavourable due to the unstable nature of the transition state formed during concerted cleavage. Instability is in part related to the size of the *O*-alkyl groups at the reaction centre of the acetal (Fife & Jao, 1965; Jensen *et al*, 1979), and thus concerted mechanisms are usually confined to acetals containing smaller *O*-alkyl groups.

The results indicate that conversion of the acetalanthraquinones to their corresponding aldehyde products was acid-catalysed, as breakdown of the acetalanthraquinones proceeded exclusively in the presence of H_3O^+ (March, 1985). Hydrolysis of the side chains is supported by the identical visible spectra of the acetalanthraquinones and their hydrolysed reaction mixtures, indicating that the chromophore remained unchanged after hydrolysis. Intramolecular cyclization of one of the alkylamino side chains to form a substituted TNQ, which can sometimes occur on heating anthraquinones to temperatures above 50°C (see section 3.3.2) would have produced a bathochromic shift in the visible region of the spectrum. This has been shown for the TNQ metabolite of mitoxantrone, which is blue shifted by 30nm (Blanz *et al*, 1991a). Furthermore, the TNQ metabolite is less polar than mitoxantrone (Mewes *et al,* 1993). None of the products generated by hydrolysis of the acetalanthraquinones were less polar than the original compound.

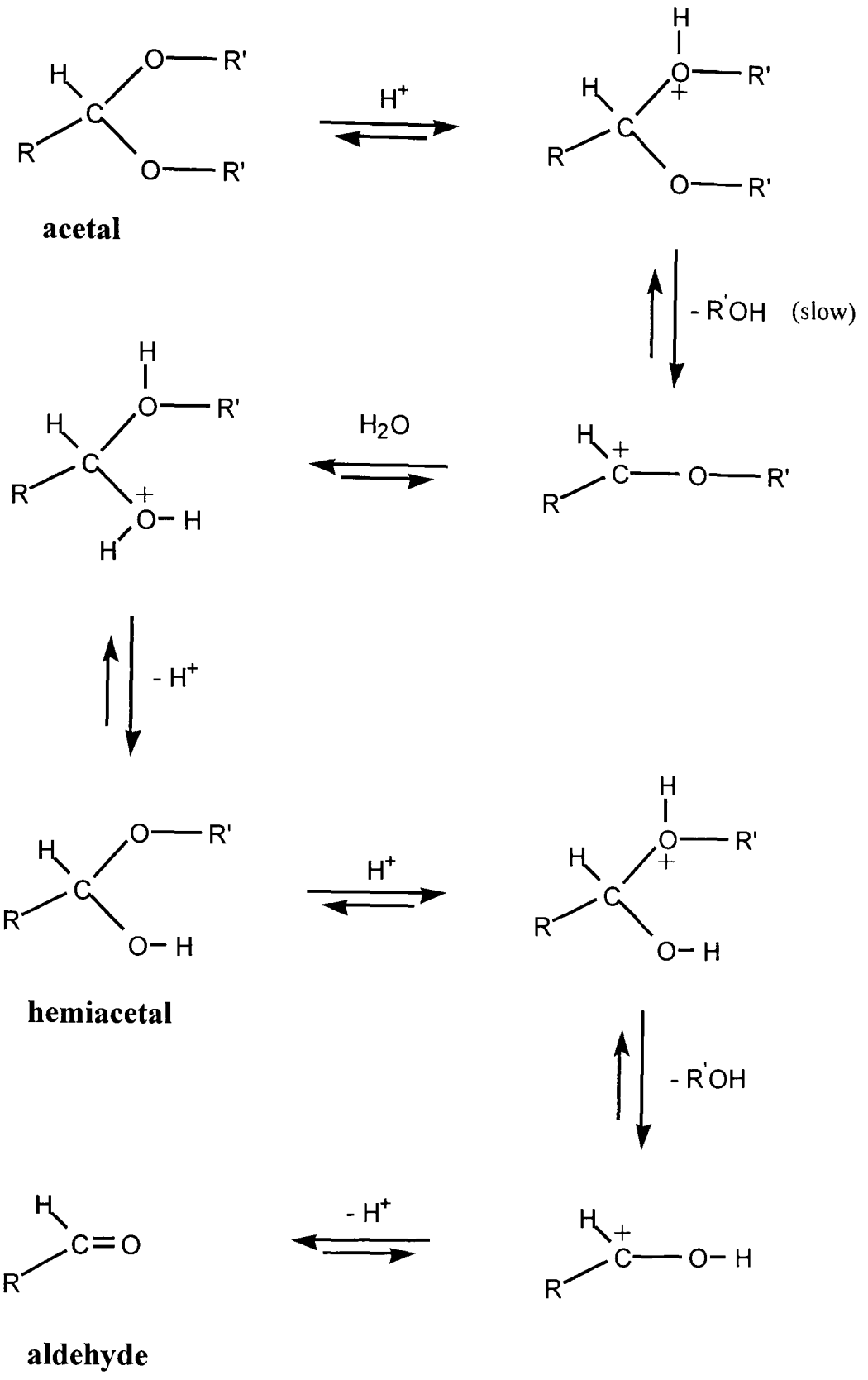


Figure 4.17: Scheme for the conversion of an acetal to the corresponding aldehyde by acid-catalysed hydrolysis, via S_N1 nucleophilic substitution.

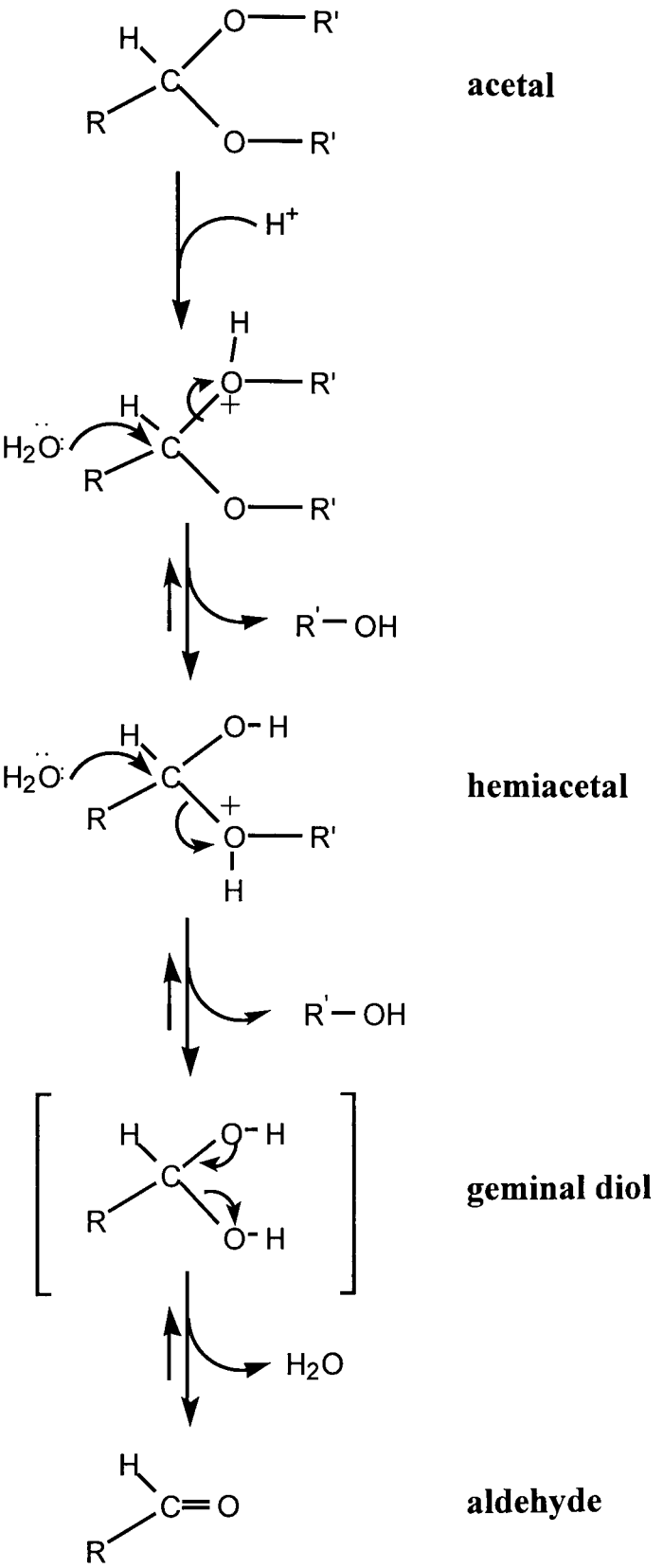


Figure 4.18: Scheme for the conversion of an acetal to the corresponding aldehyde by acid-catalysed hydrolysis, via S_N2 nucleophilic substitution.

In contrast to the visible spectra, a noticeable change occurred in the UV region of the spectrum after hydrolysis. As double bonds are known to absorb in this region, it could be argued that the generation of aldehyde groups is responsible for the increase in absorbance observed. Further oxidation of the aldehydes to their corresponding carboxylic acids seems unlikely in view of the fact that all solutions of acetalanthraquinone were protected from UV light in sealed Eppendorf tubes.

Ametantrone was found to be stable under the experimental conditions used. The ametantrone side chains would be refractory to hydrolysis due to the replacement of the *O*-methyl groups of the acetalanthraquinone side chains with primary alcohol groups, which when protonated, do not possess the electron withdrawing properties of the protonated *O*-methyl groups.

The two products yielded for YCG7, and three products yielded for both YCG8 and YCG9 all had shorter retention times than the parent compounds, consistent with the generation of aldehydes/hemiacetals. The aldehydes would be expected to be more polar than the acetalanthraquinones due to the electron-withdrawing effect of the carbonyl oxygen, increasing the hydrogen bonding capabilities of the side chains. The two products yielded for YCG7 were probably the hemiacetal and aldehyde oxidation products of the single side chain. The products for YCG8 and YCG9 were probably the parent/hemiacetal, hemiacetal/aldehyde and aldehyde/aldehyde side chain combinations (figure 4.19). The hemiacetal/hemiacetal combination would probably not be detected, as the hemiacetal species is relatively short lived, its formation from the acetal usually being the rate-determining step (Fife, 1972; Fessenden & Fessenden, 1986).

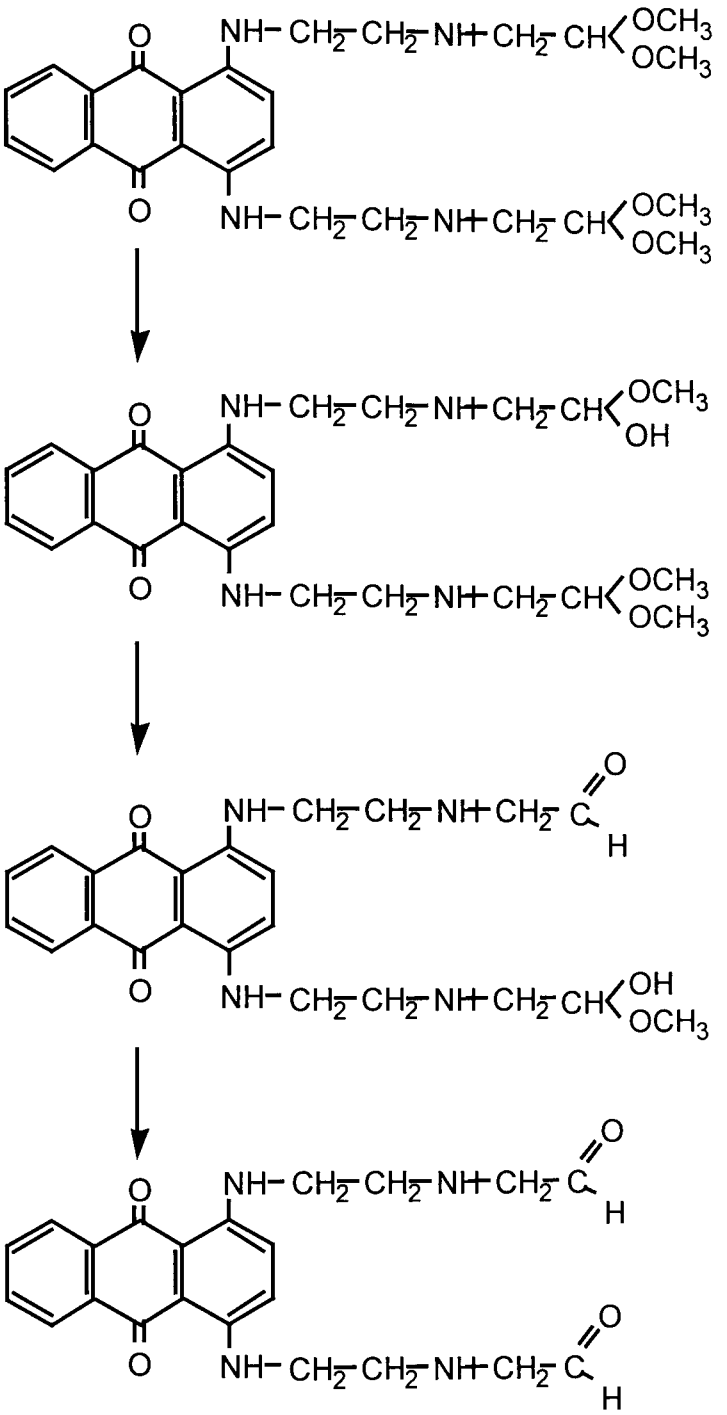


Figure 4.19: Proposed intermediates formed during the hydrolysis of YCG8 to its di-aldehyde derivative. Similar intermediates may be generated by hydrolysis of YCG7 and YCG9.

The hydrolysis rates of the acetalanthraquinones was measured from loss of the parent compound. By this calculation, hydrolysis of YCG9 appeared to be faster than for YCG7 and YCG8. The slightly slower reaction rate of YCG8 compared to YCG9 may be explained by the side chain positioning. It can be argued that as the side chains of YCG8 are in closer proximity to each other than those of YCG9, protonation of one side chain would make protonation of the other less favourable due to the slight charge repulsion this may produce. This is reflected in the slight increase in activation energy for YCG8, which to a large extent determines the reaction rate (Morris, 1985; Fessenden & Fessenden, 1986). The slower hydrolysis rate of YCG7 may have been due to its slight insolubility in water, despite the presence of DMSO.

The first order rate constants obtained would suggest that the acetalanthraquinones are hydrolysed by the S_N1 mechanism. However, considering steric effects are unlikely to affect the reaction centre of the acetalanthraquinone side chain, hydrolysis is more likely to occur by the S_N2 mechanism. Although bimolecular mechanisms usually follow second order kinetics, S_N2 reactions in which one reactant (in this case water) is in large excess, usually display *pseudo* first order kinetics (Morris, 1985; see appendix A6). In support of this, several other methoxy-acetals have been shown to undergo hydrolysis by the concerted mechanism, including 2-(p-methoxyphenyl)-4,4,5,5-tetramethyl-1,3-dioxolane (Fife, 1972).

4.4.2 OXIDATIVE METABOLISM OF THE ACETAL -ANTHRAQUINONES

The acetalanthraquinones were found to undergo aerobic metabolism, demonstrated by the formation of several products in the presence of mouse liver microsomes. Metabolite production was shown to be NADPH-dependent, indicating the conversion of the acetalanthraquinones to their metabolites was enzymically catalysed. This was confirmed by the absence of metabolism in the presence of boiled microsomes. Metabolism produced 2 products for YCG7, and 3 products for YCG8 and YCG9, which, similar to the hydrolysis products, were all more polar than the acetalanthraquinones. The rate of metabolism of YCG8 and YCG9 was essentially the same, so it can be concluded that neither the 1,4- nor the 1,5- configuration involved steric hindrance between the acetalanthraquinone side chains and the active site of the enzyme(s) involved metabolism.

The anthraquinone nucleus has been shown to be resistant to oxidation under the conditions used in this study (Wolf *et al*, 1986). Thus, it is proposed that the metabolites were produced by oxidation of the alkylamino side chains. Further evidence for this is provided by the identical absorbance spectra of the metabolic mixtures and acetalanthraquinones in the visible region of the spectrum. It is predicted that metabolism occurs via cytochrome P-450-dependent mixed function oxidation, since MFOs are abundant in mammalian liver microsomal fractions and perform a diverse range of functionalization (phase I) reactions (see section 1.5.1). The proposed route for the metabolic oxidation of the acetalanthraquinones is illustrated in figure 4.20 for YCG8. Cytochrome P-450-mediated oxidation produces a hydroxy intermediate, resulting in the loss of formaldehyde to generate an unstable hemiacetal. Subsequent protonation of the *O*-methyl group of the hemiacetal results in the spontaneous elimination of methanol to produce the aldehyde.

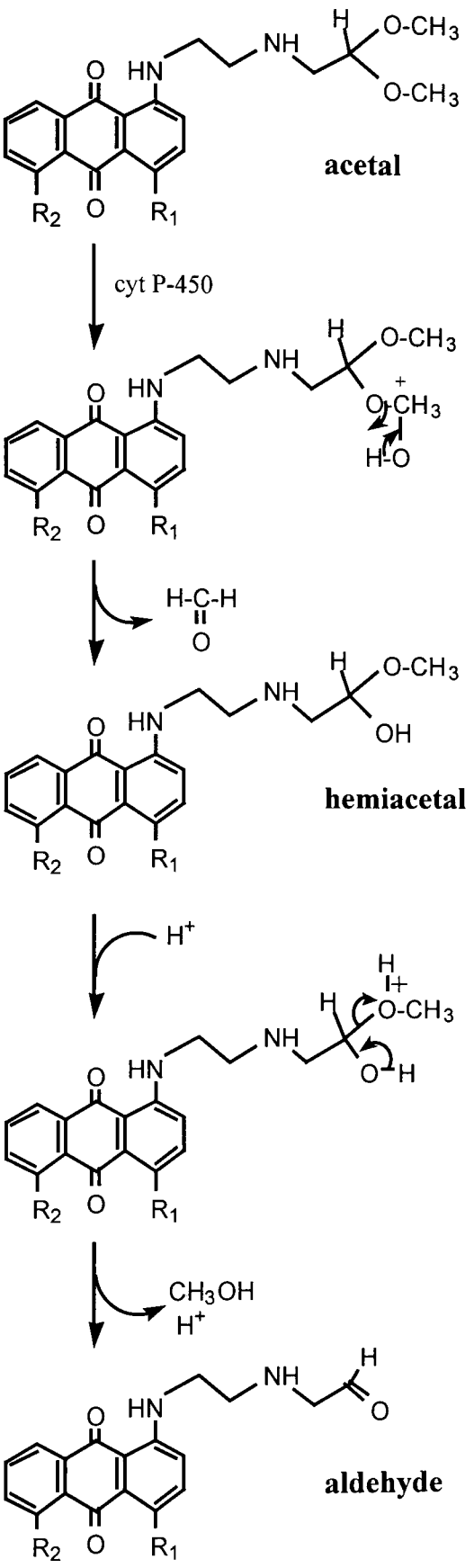


Figure 4.20: Proposed pathway for metabolic conversion of the acetalanthraquinones, via oxidation, to their corresponding aldehyde derivatives. YCG7: R1=R2=H; YCG8: R1=$NHCH_2CH_2NHCH_2CH(OCH_3)_2$; R2=H; YCG9: R1=H, R2= $NHCH_2CH_2NHCH_2CH(OCH_3)_2$.

Theoretically, the aldehyde products of the acetalanthraquinones could be oxidized to produce their corresponding mono- and dicarboxylic acids. However, as the carboxylic acid derivatives of mitoxantrone have only been detected in very small quantities in other rodent microsomal systems, if at all (Wolf *et al*, 1986; Blanz *et al*, 1991b; Richard *et al*, 1991), this seems unlikely. Furthermore, aldehyde oxidation would require the involvement of enzymes such as aldehyde dehydrogenase or aldehyde oxidase, located in the soluble (cytosolic) fraction of the liver (Gibson & Skett 1986; Beedham, 1997). It is also unlikely that the aldehydes would undergo conjugation (phase II) reactions such as sulfate- or glutathione conjugation, as these would be catalysed by enzymes not predominantly found in microsomal liver fractions, and would require the presence of the corresponding cofactors (see Gibson & Skett, 1986). Conjugation reactions are also far more likely to participate in the inactivation of cytotoxic agents rather than enhancing their activity, as is the case for cyclophosphamide (Ohno & Ormstad, 1985; Lebsanft *et al*, 1989). On the contrary, metabolism of the acetalanthraquinones was found to enhance their activity (see section 5.3.2.4).

The increased affinity of the metabolized drug for microsomal proteins observed only in incubates containing NADPH also supports the hypothesis that the drugs were metabolized to one or more species that could react covalently. If this species was an aldehyde, it would be capable of reacting with nucleophiles such as primary amines present in the microsomal protein, forming Schiff's bases (see section 5.4.2). This is further indicated by the fact that a proportion of this drug remained bound after the addition of acetonitrile as non-covalent binding should be disrupted under these conditions. Similar observations were made by Richard *et al* (1991), after metabolism of mitoxantrone using hepatocytes and by Lau *et al* (1989) after metabolism of morpholino doxorubicin. In addition, cytochrome P-450-mediated covalent binding of etoposide to liver and HeLa cell microsomal proteins has been observed by Van Maanen *et al* (1987) and Haim *et al* (1987). It is thought that *O*-demethylation of the dimethoxyphenol ring

is probably involved in the binding process (Van Maanen *et al*, 1985a). The product of *O*-demethylation has been identified as the *ortho*-dihydroxy derivative (Van Maanen *et al*, 1985b).

In conclusion, acid-catalysed hydrolysis and oxidative metabolism appear to provide effective routes for the oxidation of acetalanthraquinones, probably generating aldehydes. It is feasible that hydrolysis occurs by the S_N2 mechanism, as steric effects are unlikely to affect the reaction centre of the acetalanthraquinone side chain. Metabolic conversion of the acetalanthraquinones was achieved by an NADPH-dependent oxidative process which is likely to involve one or more isoforms of cytochrome P-450.

CHAPTER 5: DNA BINDING AND CYTOTOXICITY OF ACETALANTHRAQUINONES

5.1 INTRODUCTION

DNA intercalating agents such as the anthraquinones and anthracyclines have a well established role in the treatment of a wide variety of human cancers. Their cytotoxic effects are thought to be mediated via several mechanisms, predominantly involving DNA intercalation and inhibition of topoisomerase II (see sections 1.2.1, 1.2.2 and 1.3.2). There is evidence that the DNA damage caused by agents that stabilize topoisomerase II cleavable complexes results from the interaction of these complexes with DNA replication and transcription forks, producing chromosome aberrations and genetic recombination events (see section 1.3.2.3). This commonly results in cell cycle arrest in either the G1 or G2 phases, providing an opportunity for the DNA damage to be repaired (Wassermann, 1994; Chaney & Sancar, 1996). In cells where damage is unrepaired or mis-repaired, the cell ultimately responds by triggering cell death via apoptosis (Hickman, 1992).

Various attempts have been made to improve the cytotoxic activity (and minimize the side effects) of anticancer agents by chemical or biological activation. Activated drugs with increased cytotoxic potency include the N-oxide AQ4N, doxorubicin analogues (e.g. mdox, mmdox) and cyclophosphamide (see sections 1.5.2-1.5.4). In the previous chapter, it was shown that the acetalanthraquinones were converted via hydrolytic and metabolic pathways to several other compounds, one or more of which appeared to have an increased affinity for proteins (see sections 4.3.2, 4.3.3 and 4.3.4). It follows that if these products are the corresponding aldehyde derivatives, they should be more reactive than the acetals due to the potential for Schiff's base formation with intracellular enzymes, which may include topoisomerase II. With this in mind, an investigation was made into the DNA binding and cytotoxic activity of the acetalanthraquinones and their products.

5.2 METHODS AND MATERIALS

Acetalanthraquinones were synthesized as described in sections 3.2.2.1-3.2.2.3. All sterile cell culture materials were supplied by Flowlabs, Hertfordshire, UK, unless otherwise stated. Reagents were supplied by Sigma Chemical Co. Poole, Dorset, UK, unless otherwise stated. RPMI 1640 medium was prepared as described in appendix A7.1. The medium was supplemented with 10% foetal calf serum, 200mM L-glutamine (1% v/v) and 1% v/v penicillin (5000IU/ml)/streptomycin (5000μg/ml) and was pre-warmed to 37°C before use. This was used for all experiments and has been referred to as 'fully supplemented'. The V79 Chinese hamster lung cell line (CHL cell line; ECACC No 86041102) was derived from cells spontaneously transformed from the lung fibroblasts of the Chinese hamster. Solutions of drug used for cytotoxicity studies were sterile filtered using 0.2μm flowpore syringe filters, with the exception of those used in the drug metabolism studies. Bacterial contamination was avoided in such cases by doubling the penicillin/streptomycin concentration of the RPMI medium during and after the drug incubation period. Solutions of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were stored at 4°C and used within 1 week. The procedure used to determine the effect of DNA on the spectral properties of acetalanthraquinones was as described in section 2.2.2.1. Where abbreviations have been used for reagents, full names can be found in appendix A8.

5.2.1 DNA BINDING STUDIES

5.2.1.1 THE EFFECT OF ACETALANTHRAQUINONES ON THE THERMAL DENATURATION OF CALF THYMUS DNA

The effect of the acetalanthraquinones on the thermal denaturation of DNA before and after hydrolysis was investigated. YCG 7 was hydrolysed at 90°C for 12 h and YCG8 and YCG9 were hydrolysed at 90°C for 9 h as described in section 4.2.2. The drugs were then neutralized with acetone-ice-cold 0.1M NaOH and extracted into dichloromethane. The solvent was removed by evaporation *in vacuo* and determination

of Tm was carried out on the acetalanthraquinones and the hydrolysed products by the procedure described in section 2.2.2.3. An identical procedure was carried out with ametantrone.

5.2.2 CYTOTOXICITY STUDIES

5.2.2.1 MAINTENANCE OF V79 CELLS

V79 cells, which grow in monolayer culture, were grown in fully supplemented RPMI 1640 medium in $150cm^3$ flasks (Corning, NY, USA) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Cells were passaged twice weekly using EDTA (0.1% in DPBS) as described in appendix A7.2.

5.2.2.2 MTT FORMAZAN CALIBRATION CURVE

MTT formazan (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenyl-formazan) was dissolved in DMSO to give solutions ranging in concentration from 5 to 100μgml^{-1}. Aliquots (8 x 100µl) at each concentration, were pipetted into 96-well microtitre plates. The absorbance was determined against a DMSO blank at 492nm on a Titertek multiscan MKII microtitre plate reader (MCC/340 MKII). MTT formazan concentration was plotted versus absorbance at 492nm to ensure linearity.

5.2.2.3 CALIBRATION OF MTT FORMAZAN PRODUCTION AND V79 CELL DENSITY

V79 cells in exponential growth were passaged and collected in approximately 5ml of fully supplemented RPMI 1640 culture medium. The resulting cell suspension was passed through a 19G needle two or three times to disperse cell clumps and the cell concentration determined using an electronic particle counter (Coulter Electronics Ltd; see appendix A7.3). The cell suspension was adjusted with fully supplemented RPMI medium to give cell densities between 1 x 10^4 and 6 x 10^6 cells/ml. At each cell density, 8 x 100µl was dispensed into 96-well microtitre plates (leaving one column free on each

plate for the blank), sealed in polythene and incubated at 37°C for 4 h to allow cells to adhere to the plates. The RPMI medium was then removed by inversion and gentle tapping of the plates, and 25µl of pre-warmed, sterile filtered MTT solution (2mg/ml) was added to each well. After a further 4 h incubation at 37°C, the resulting MTT formazan crystals were solubilized by gentle shaking with DMSO (100µl per well) for 30 min on a vortex mixer. The absorbance was measured at 492nm versus a blank of MTT (2mg/ml; 25µl per well) and DMSO (100µl/well). The absorbance at 492nm (equivalent to MTT formazan production) was plotted against V79 cell density to ensure linearity.

5.2.2.4 DETERMINATION OF V79 CELL GROWTH CHARACTERISTICS AND CELL DOUBLING TIME

V79 cells in exponential growth were passaged and collected in fully-supplemented RPMI 1640 medium and passed through a 19G needle two or three times. The cell concentration was determined using a particle counter and the suspension was diluted in medium to give cell densities of 1×10^4 to 1×10^6 cells/ml. Cell suspension (8 x 100µl per concentration) was added to 96-well microtitre plates, sealed in polythene and incubated at 37°C for 4, 24, 48, 72, 96 and 120 h. The culture medium was changed every one or two days, where necessary, by inversion and tapping. After the appropriate time had elapsed, the medium was removed, 25µl of pre-warmed, sterile filtered MTT solution (2mg/ml) was added to each well and the cells were incubated for a further 4 h at 37°C. The resulting MTT formazan product was then solubilized, and absorbance measurements taken as described in section 5.2.2.2. The cell doubling time was calculated by linear regression, from a plot of cell number versus time.

5.2.2.5 EFFECT OF ACETALANTHRAQUINONES ON V79 CELL VIABILITY

V79 cells in exponential growth were passaged, collected in pre-warmed, fully-supplemented RPMI 1640 medium and counted using an electronic particle counter. The cell suspension was adjusted to 7.5 x 10^4 cells/ml with RPMI medium and 100µl aliquots pipetted into all but the last column of each 96-well microtitre plate. After incubation at 37°C for 48 h, the culture medium was removed by inversion and tapping, and replaced with fully-supplemented RPMI medium, containing the appropriate concentration of sterile filtered acetalanthraquinone, (100µl/well). Medium (100µl), containing the appropriate volume of water in place of drug solution, was added to the eight wells serving as the control column. Following 4 or 24 h incubation at 37°C, the solutions were removed, and each well washed three times with pre-warmed DPBS. The cells were replenished with 100µl pre-warmed, fully-supplemented medium and subsequently left to grow at 37°C for a further 72 h, replacing exhausted medium when necessary. After 72 h, the culture medium was removed and the cell survival at each drug concentration was then determined by the MTT assay, as described in section 5.2.2.2. Cytotoxicity was expressed as the concentration of drug required to reduce the viability of the cell population by 50% (EC_{50}). The above procedure was repeated using drug solutions prepared in culture medium diluted with sterile distilled water (10% v/v) instead of foetal calf serum.

5.2.2.6 EFFECT OF ACID-HYDROLYSED ACETALANTHRAQUINONES ON V79 CELL VIABILITY

The procedure was as described in section 5.2.2.5, except that prior to incubation with V79 cells, the acetalanthraquinones were hydrolysed in 0.1M HCl at 90°C for 9 h (YCG8 and YCG9) and 12 h (YCG7) then neutralized with 0.1M NaOH and cooled on ice-acetone (see section 4.2.2 for experimental detail). The procedure was also performed with drug maintained at 37°C in 0.1M HCl. Drug solutions were then diluted in RPMI 1640 medium (with or without foetal calf serum, 10% v/v) to give drug

concentrations between 1.0 and 10μM. The effect of HCl/NaOH on cell viability was also investigated.

5.2.2.7 EFFECT OF ACETALANTHRAQUINONE METABOLITES ON V79 CELL VIABILITY

For YCG8 and YCG9, metabolism was carried out for 60 min at 37°C. As the metabolic products of YCG7 were formed more slowly than those of the bis-substituted compounds, metabolism of this compound was carried out for 70 min in order to obtain the same proportion of metabolites for all three compounds (50 ± 5%). This was essential if the cytotoxicity results were to be comparable. Two sets of control incubations were also set up, the first consisting of drug incubated for 60 (or 70) min in the absence of NADPH, and the other containing NADPH but no drug. The procedure for metabolism was carried out as described in section 4.2.3, except that instead of adding acetonitrile to the samples prior to spinning down the microsomal protein after the incubation period, samples were immediately cooled on ice-acetone for 5 min and centrifuged at 14 000 x g for 15 min. A proportion (~100μl) of each incubate was removed and analysed by HPLC to verify the formation of metabolites. The remainder of the supernatant was serially diluted in pre-warmed RPMI 1640 medium supplemented with foetal calf serum, 10% v/v, 200mM L-glutamine, 1% v/v and 2%v/v penicillin (5000IU/ml)/streptomycin (5000mg/ml) to give concentrations of acetalanthraquinone between 0.5 and 50μM. Metabolized and non-metabolized drug solutions (8 x 100μl) were incubated with V79 cells (7.5 x 10^4 cells/ml) for 4 h. The drug was then removed and the cells were washed three times with pre-warmed DPBS, then replenished with medium (penicillin/streptomycin 2%v/v) and incubated at 37°C for 72 h. The cell survival at each drug concentration was determined by the MTT assay, as described in section 5.2.2.2. The effect of 2% penicillin/streptomycin on cell viability was also investigated, as was the effect of microsomal suspension/NADPH.

5.3 RESULTS

5.3.1 DNA BINDING STUDIES

Figures 5.1 to 5.6 show spectral shifts for the acetalanthraquinones in the presence of calf thymus DNA at ionic strengths of 0.5M and 0.05M NaCl. Data are summarized in table 5.1. The acetalanthraquinone YCG7 gave a hypochromic and a slight bathochromic shift in the presence of DNA but displayed no isosbestic point either at 0.5M or 0.05M NaCl, although the spectra all converged at 415.8 and 547.3nm (0.5M) and 405.2 (0.05M). YCG8 and YCG9 both showed bathochromic and hypochromic shifts in the presence of DNA, although these were relatively small compared to those for ametantrone. YCG8 showed isosbestic points at 636.5nm (0.5M NaCl) and 639.7nm (0.05M NaCl) but YCG9 behaved in much the same way as YCG7 in that it showed no isosbestic point at either concentration, although the spectra converged at 571.3nm (0.5M). All three compounds caused an increase in the melting temperature of DNA, as shown in figures 5.8, 5.10 and 5.12, but it can be seen that this was very small for YCG7 and only moderate for YCG8 and YCG9 compared with ametantrone (figure 5.7; table 5.2). After hydrolysis, there was no change in Tm for any of the acetalanthraquinones (figures 5.9, 5.11, 5.13).

Table 5.1: Spectral properties of acetalanthraquinones in the presence of calf thymus DNA

DRUG	isosbestic point (nm)	shift in λmax (nm)[a]	% decrease in extinction[b]
ametantrone	636	12	16
YCG7	----	1.4	7
YCG8	637	3.5	10
YCG9	----	3.6	17

a: shift in λmax at a DNA to drug ratio of 10:1; b: difference between λmax of free drug and fully bound drug (hypochromic shift). All experiments were performed in 0.5M NaCl/ 0.008M Tris buffer.

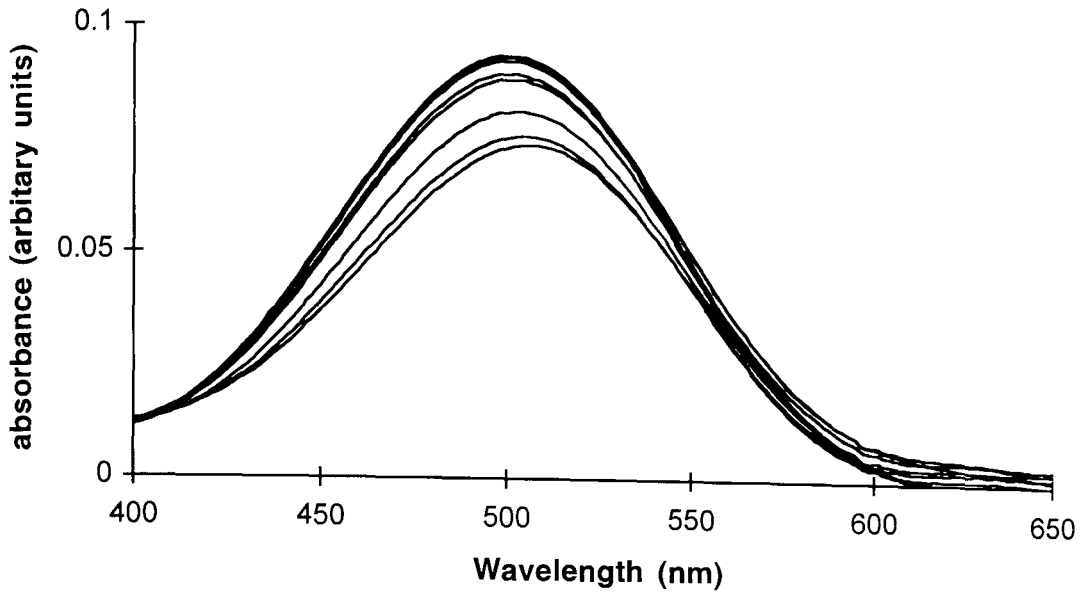


Figure 5.1: Spectral properties of YCG7 in the presence of calf thymus DNA and 0.008M Tris/0.05M NaCl.

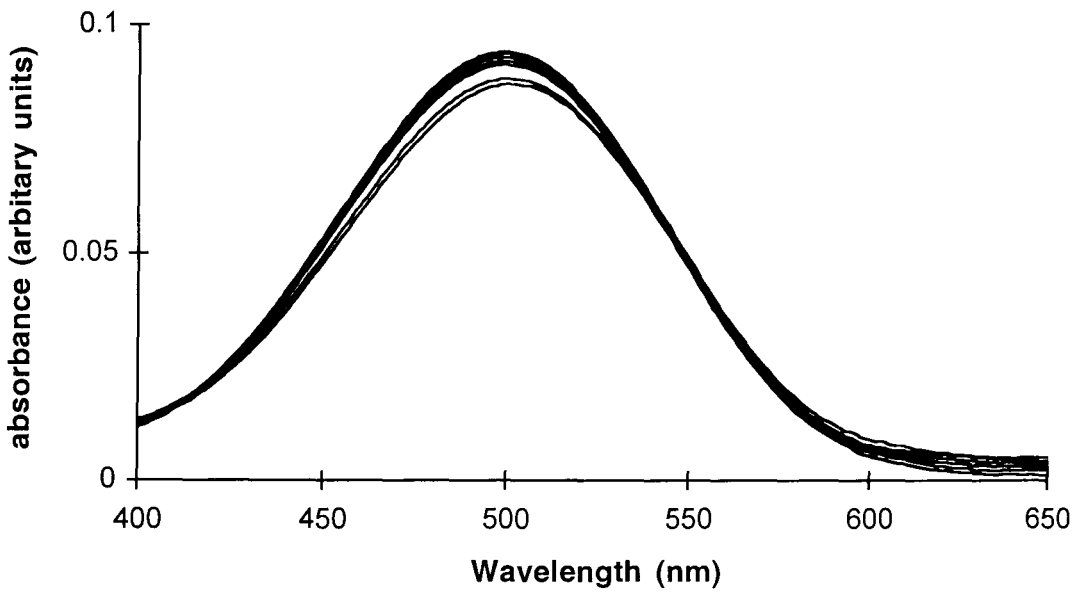


Figure 5.2: Spectral properties of YCG7 in the presence of calf thymus DNA and 0.008M Tris/0.5M NaCl.

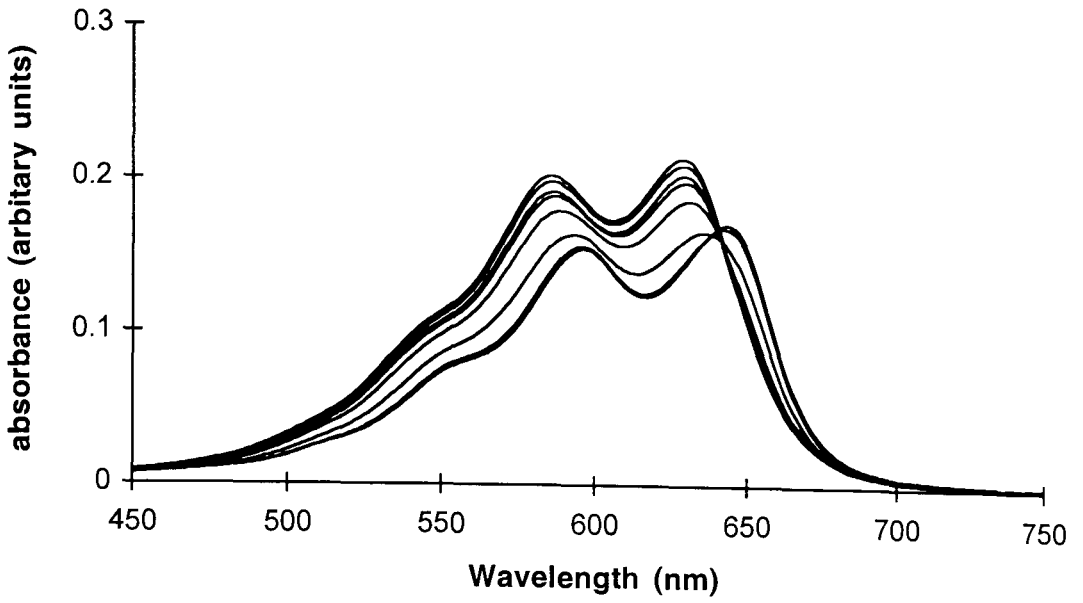


Figure 5.3: Spectral properties of YCG8 in the presence of calf thymus DNA and 0.008M Tris/0.05M NaCl.

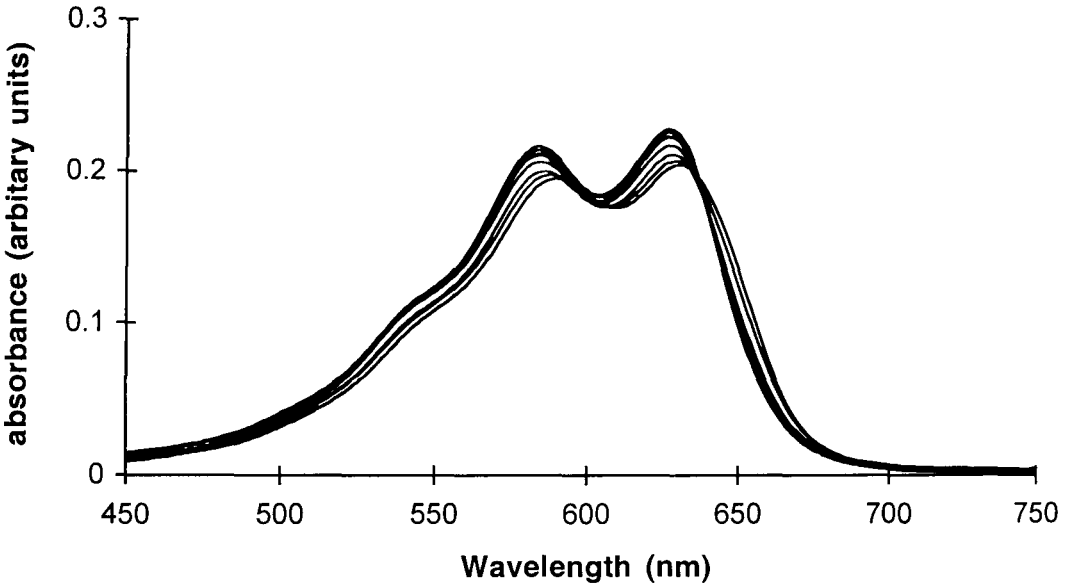


Figure 5.4: Spectral properties of YCG8 in the presence of calf thymus DNA and 0.008M Tris/0.5M NaCl.

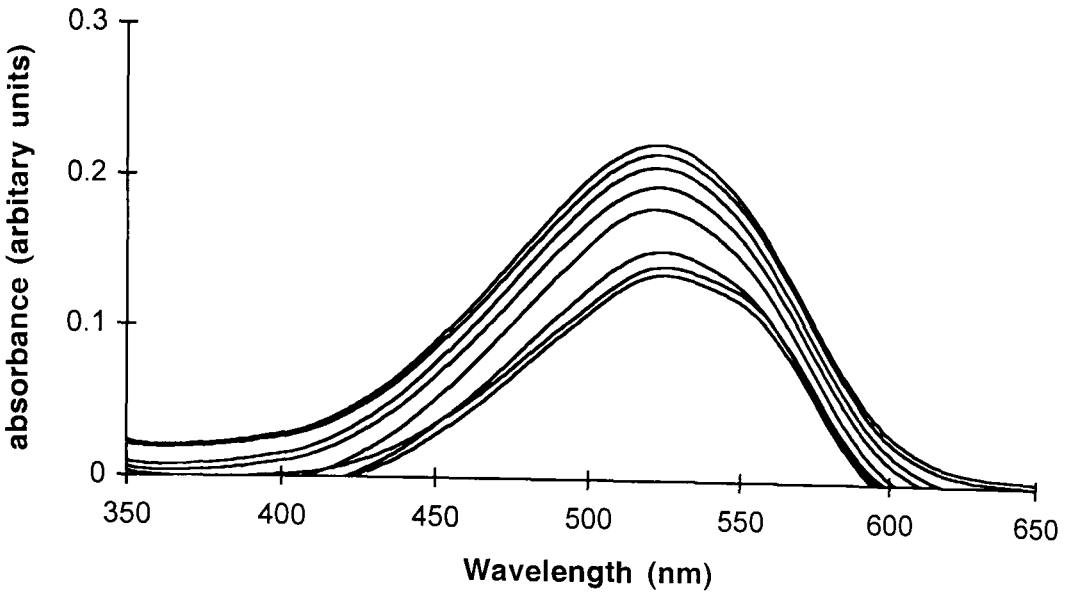


Figure 5.5: Spectral properties of YCG9 in the presence of calf thymus DNA and 0.008M Tris/0.05M NaCl.

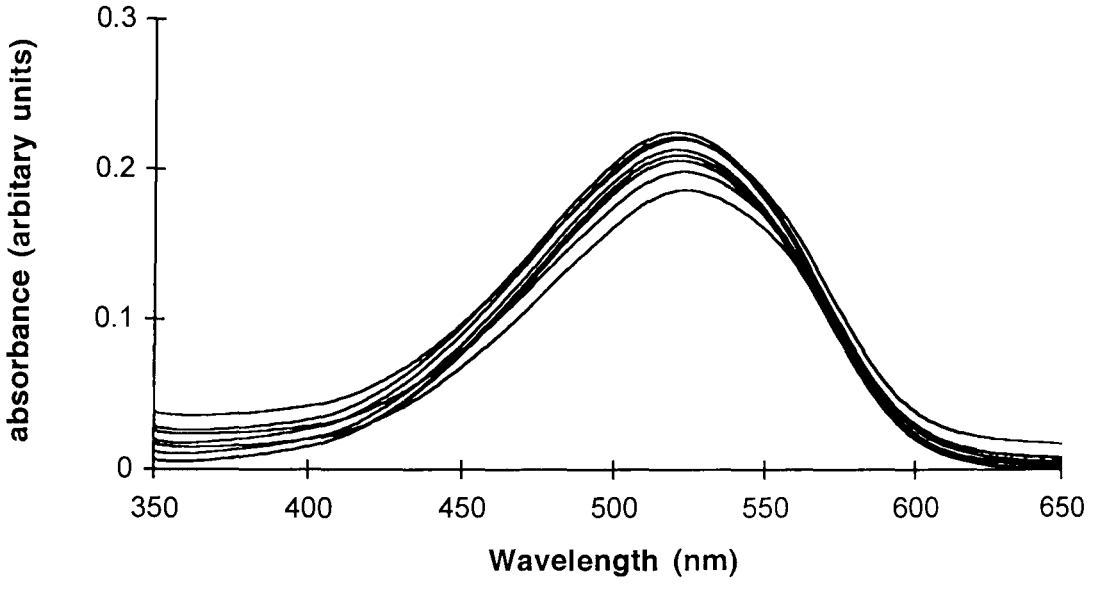


Figure 5.6: Spectral properties of YCG9 in the presence of calf thymus DNA and 0.008M Tris/0.5M NaCl.

Table 5.2: ΔTm values of calf thymus DNA in the presence of the acetalanthraquinones.

DRUG	ΔTm - hydrolysis [a] (°C)	ΔTm +hydrolysis [b] (°C)	increase in ΔTm after hydrolysis [c] (°C)
ametantrone	21.6 ± 0.1	21.6 ± 0.04	+0.04
YCG7	3.6 ± 0.03	6.1 ± 0.6	+2.5
YCG8	14.2 ± 0.1	13.5 ± 0.4	-0.7
YCG9	15.7 ± 0.4	16.5 ± 0.1	+0.8

Values were determined (a) pre- and (b) post- incubation with 0.1M HCl at 90°C. ΔTm is the difference in melting temperature of DNA in the presence and absence of drug (Tm of calf thymus DNA= 70.6 ± 0.5°C). c represents the increase in ΔTm of hydrolysed drug compared with non-hydrolysed drug. Values represent the mean ± sd for three separate experiments.

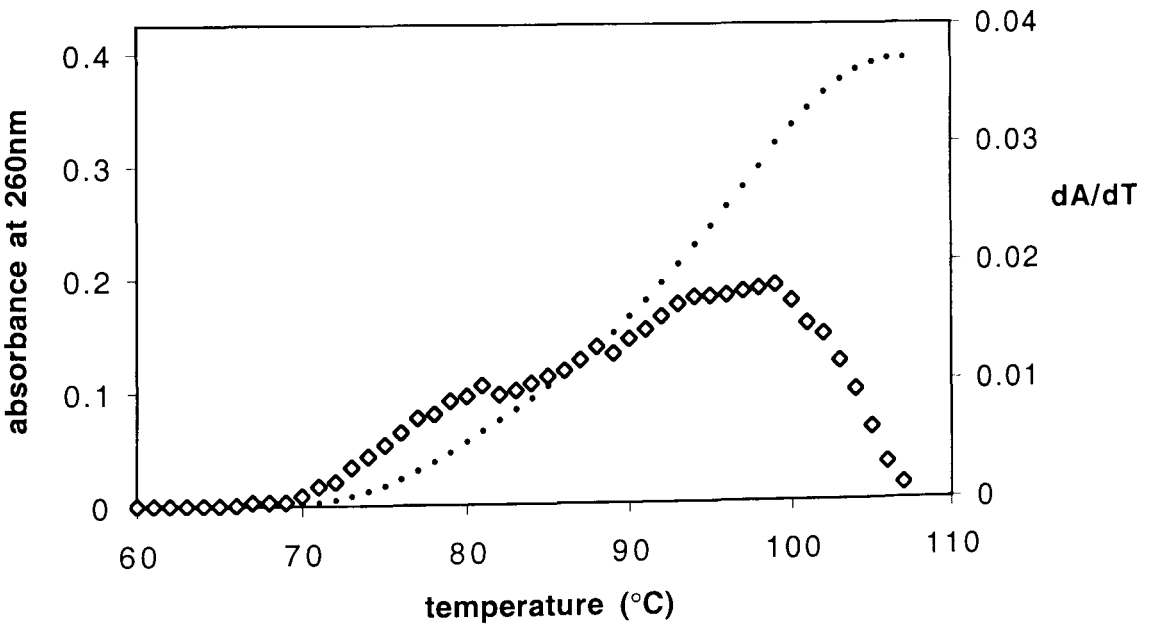


Figure 5.7: Melting profile of calf thymus DNA in the presence of ametantrone. (•) A vs T; (◊) dA/dT vs T. Values shown represent the mean of three separate experiments.

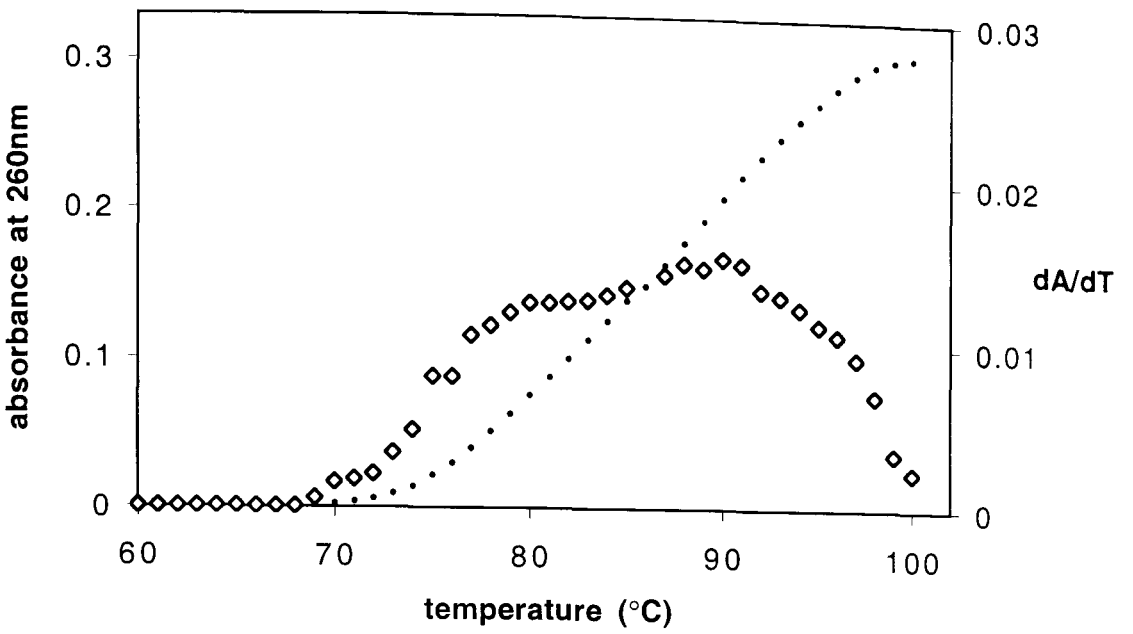


Figure 5.8: Melting profile of calf thymus DNA in the presence of YCG7 before drug hydrolysis. (•) A vs T; (◊) dA/dT vs T. Values shown represent the mean of three separate experiments.

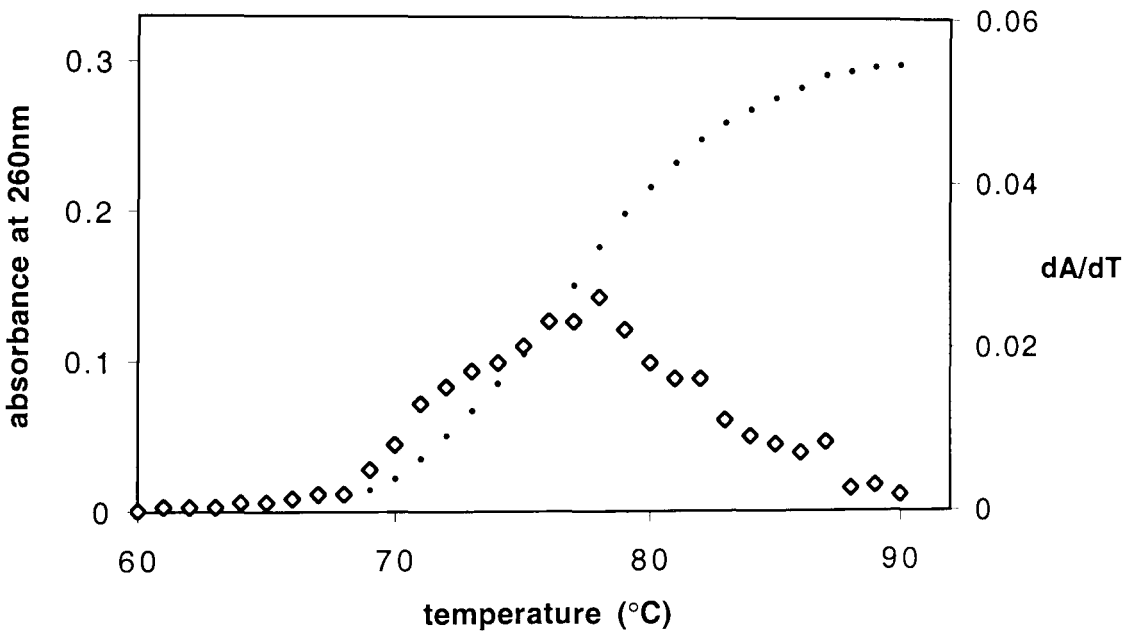


Figure 5.9: Melting profile of calf thymus DNA in the presence of YCG7 after drug hydrolysis. (•) A vs T; (◊) dA/dT vs T. Values shown represent the mean of three experiments.

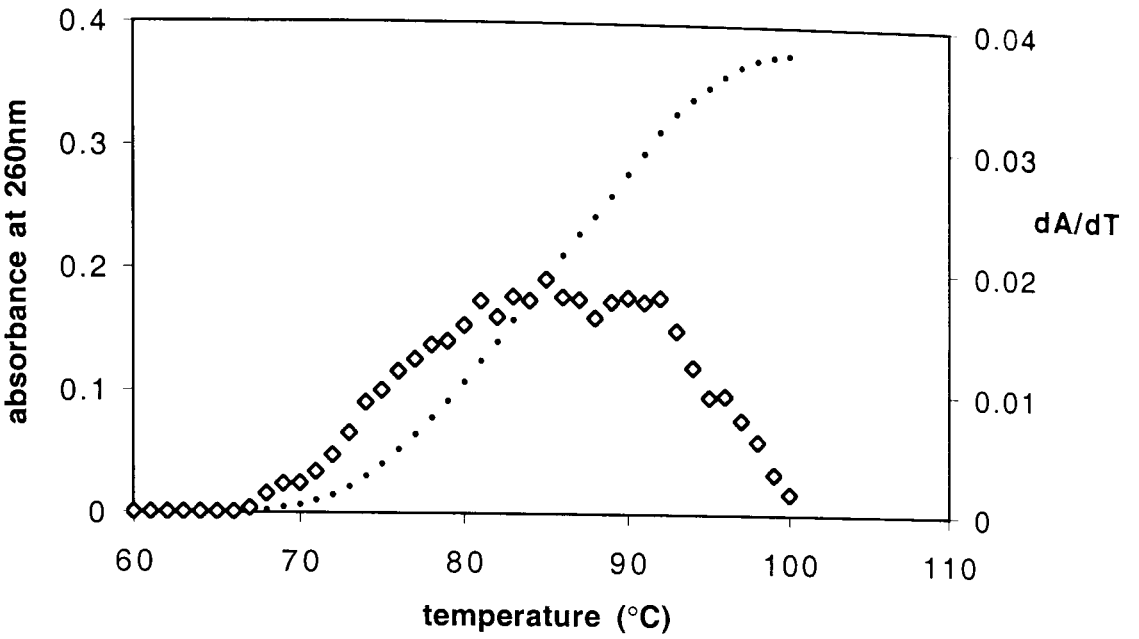


Figure 5.10: Melting profile of calf thymus DNA in the presence of YCG8 before drug hydrolysis. (•) A vs T; (◊) dA/dT vs T. Values shown represent the mean of three separate experiments.

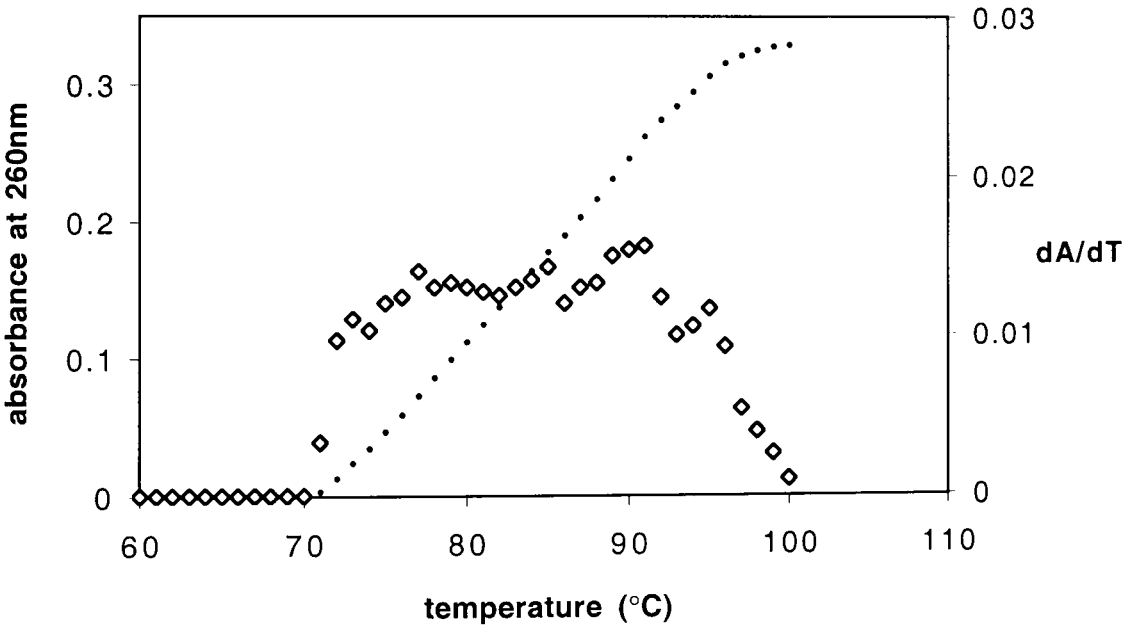


Figure 5.11: Melting profile of calf thymus DNA in the presecne of YCG8 after drug hydrolysis. (•) A vs T; (◊) dA/dT vs T. Values shown represent the mean of three separate experiments.

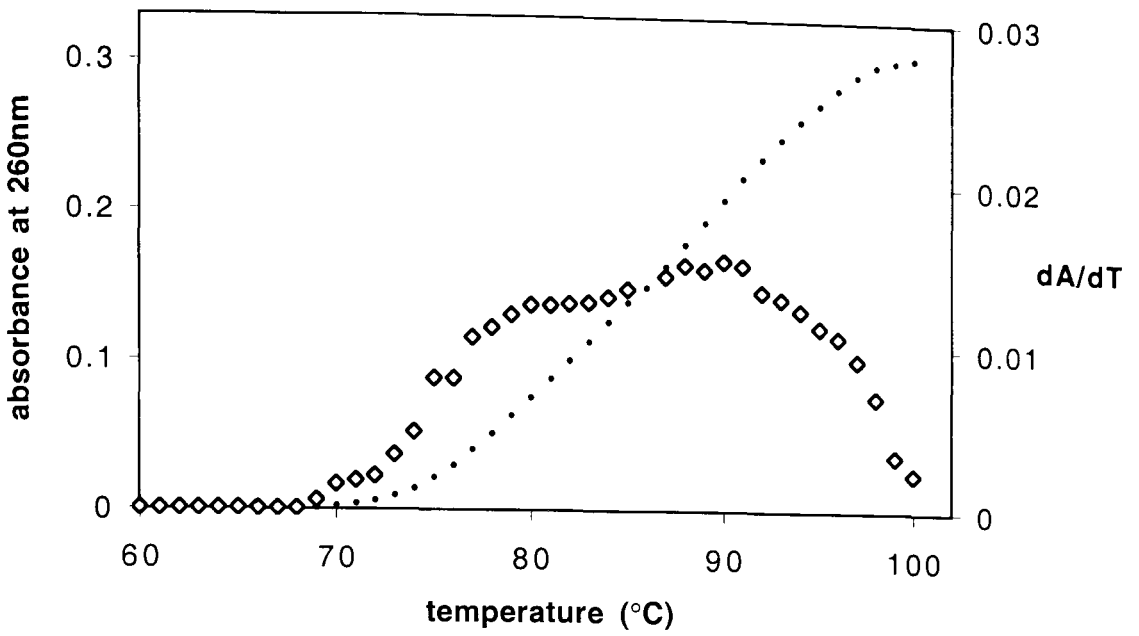


Figure 5.12: Melting profile of calf thymus DNA in the presence of YCG9 before drug hydrolysis. (•) A vs T; (◊) dA/dT vs T. Values shown represent the mean of three separate experiments.

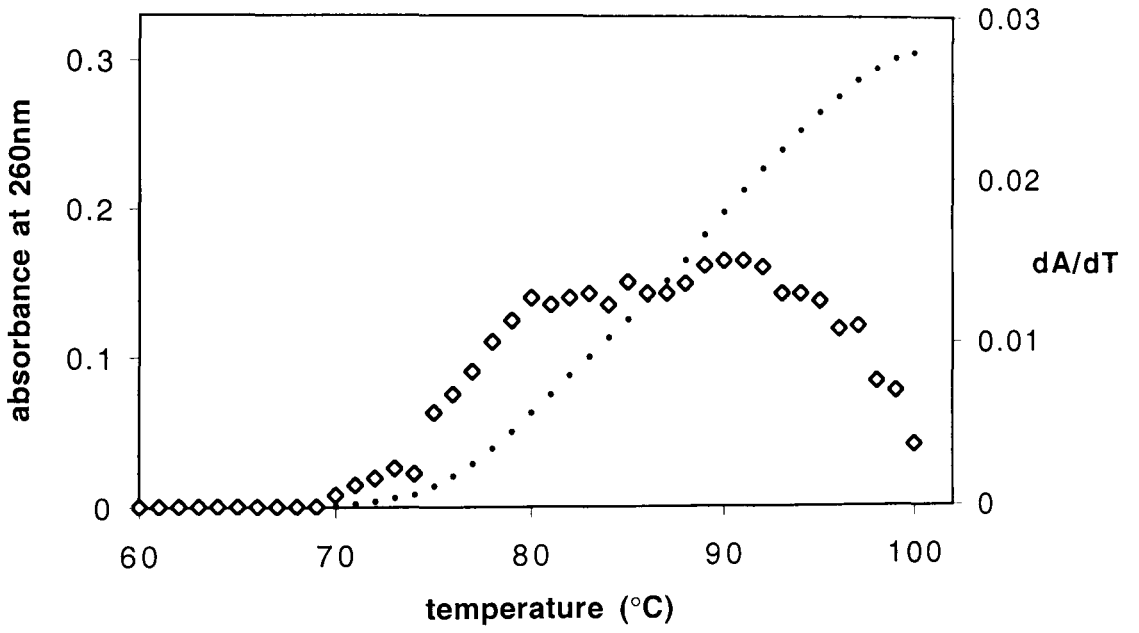


Figure 5.13: Melting profile of calf thymus DNA in the presence of YCG9 after drug hydrolysis. (•) A vs T; (◊) dA/dT vs T. Values shown represent the mean of three separate experiments.

5.3.2 CYTOTOXICITY STUDIES

5.3.2.1 THE MTT ASSAY

The MTT colourimetric assay, first used by Mosmann (1983), has proved to be a rapid, sensitive and reproducible method for the screening of potential chemotherapeutic agents in a wide variety of tumour cell types (Carmichael *et al*, 1987; Park *et al*, 1987; Alley et *al*, 1988). The assay is based on the ability of mitochondrial succinate dehydrogenase in viable cells to reduce the tetrazolium salt MTT to a purple, water-insoluble formazan product. The quantity of formazan produced thus provides an indication of the number of viable living cells in culture. As the cell membrane is impermeable to the product, it accumulates within the cell. Following addition of DMSO, the product is liberated and the absorbance can be measured.

The relationship between MTT formazan concentration and absorbance at 492nm was found to be linear between 0 and 100µg/ml (figure 5.14). V79 cell density was proportional to the formation of MTT formazan between 1 x 10^4 to 6 x 10^6 cells/ml (figure 5.15). Figure 5.16 shows V79 cell growth characteristics at initial seeding densities between 1x10^4 and 1x10^6 cells/ml over a 5 day period. At lower seeding densities (1.0 x 10^4 - 7.5 x 10^4 cells/ml) there was a 24 h recovery (lag) phase before the onset of exponential growth. At higher cell concentrations (1 x 10^5 - 1 x 10^6 cells/ml), there was no apparent recovery phase and the cell growth began to reach a plateau after 48-72 h. The cell doubling time was 18.3 ± 3.9 h, in agreement with that of ~ 18 h previously sited by Suzuki & Nakane (1994).

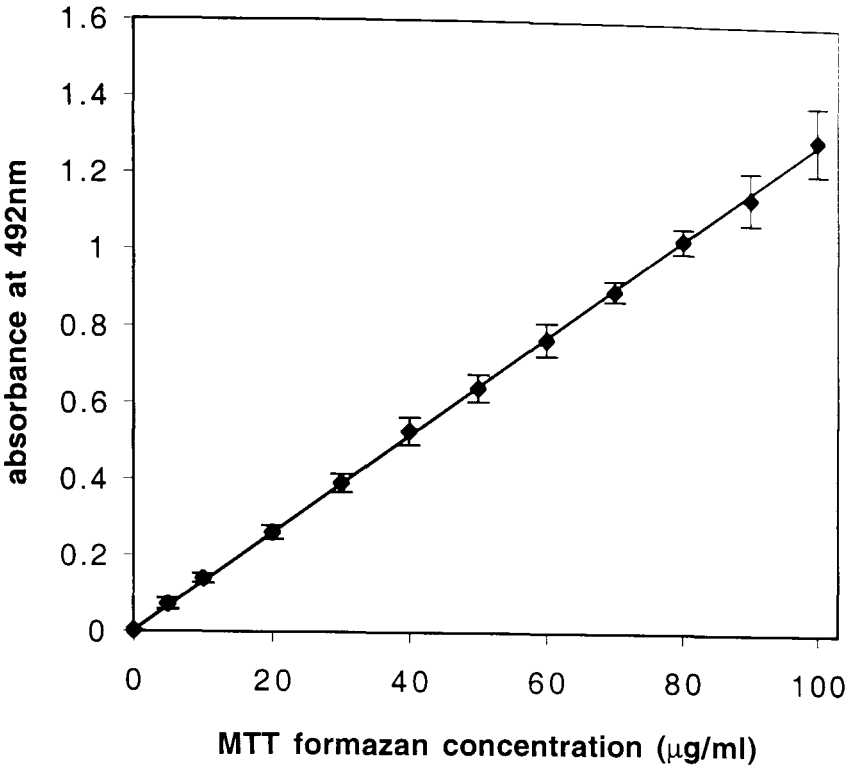


Figure 5.14: Relationship between MTT formazan concentration and absorbance at 492nm. Values shown represent the mean ± sd of three separate experiments.

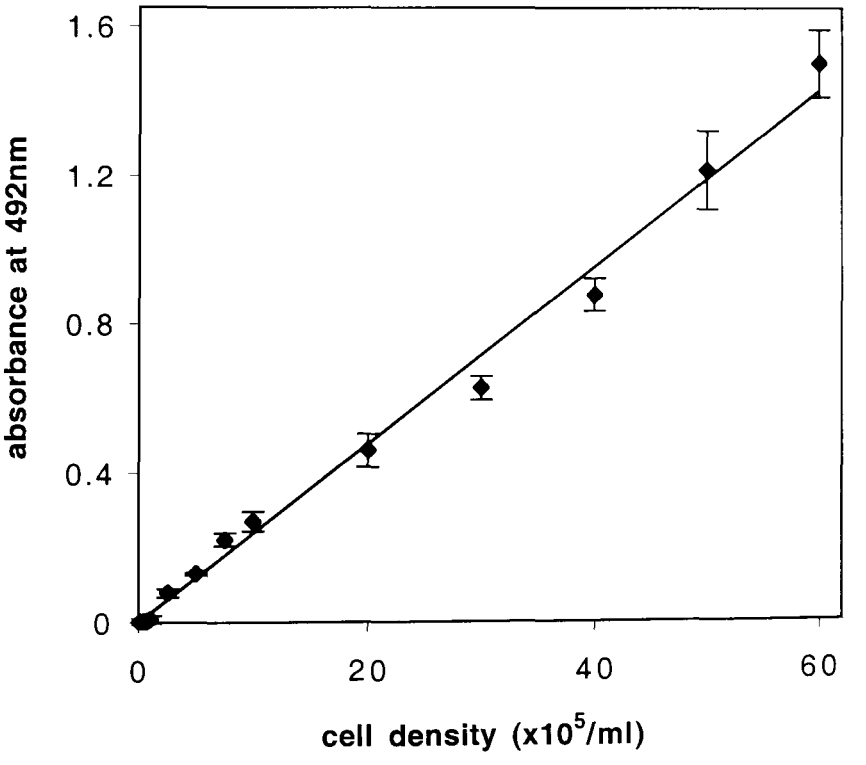


Figure 5.15: Relationship between V79 cell density and MTT formazan production. Values shown represent the mean ± sd of three separate experiments.

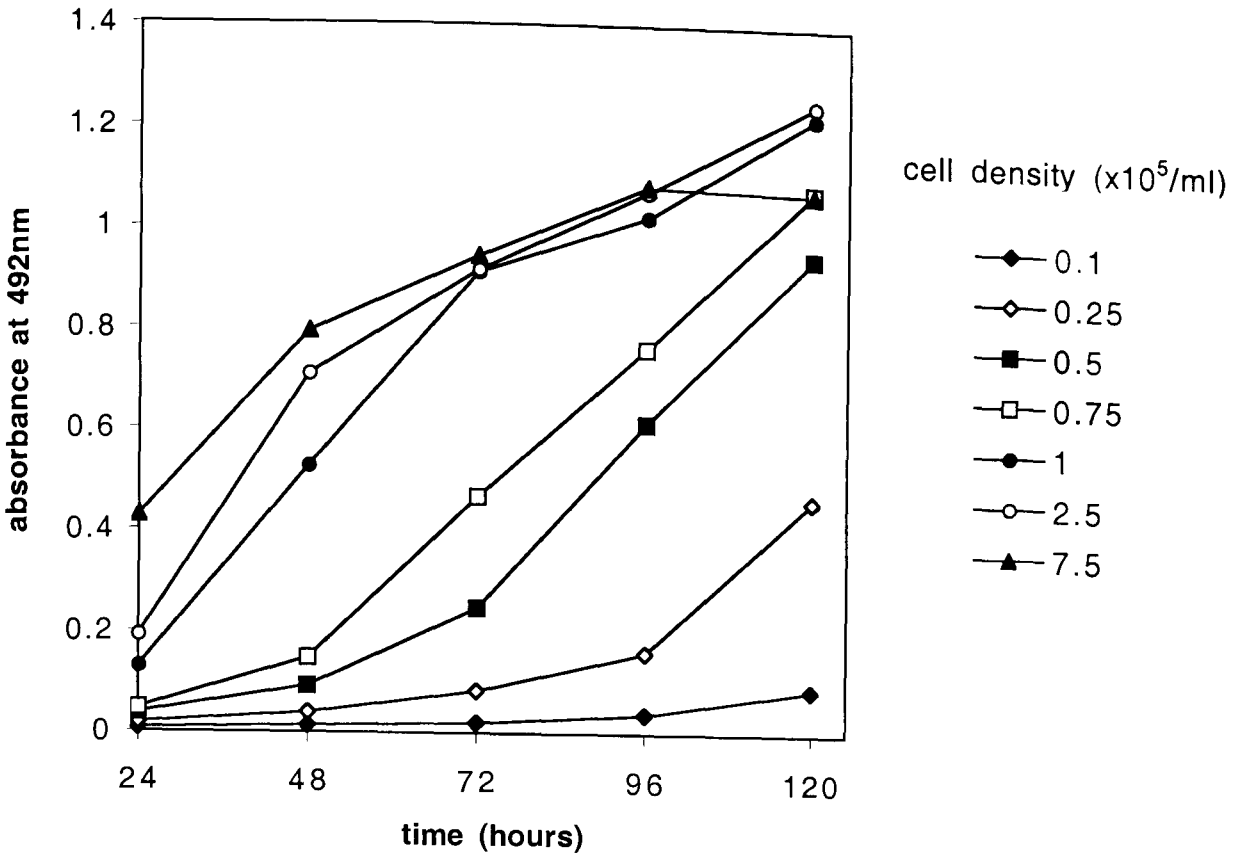


Figure 5.16: Growth characteristics of V79 cells, seeded at various densities over a 5 day period. The values shown represent the mean of three separate experiments.

The linearity obtained between MTT formazan production (R^2=1.0) and V79 cell density up to 6 x 10^6 cells/ml (R^2=0.998) and the consistency of the values (standard deviations < ±10%) both confirmed that this assay was suitable for determining the effect of the acetalanthraquinones and their products on V79 cell proliferation. An inoculation density of 7.5 x 10^4 cells/ml (7.5 x 10^3 cells/well) was deemed to be most suitable for subsequent cytotoxicity studies due to the growth characteristics at this density. Cells were grown for two days prior to drug incubation to ensure exponential growth, and for a further three days post-incubation.

5.3.2.2 THE EFFECT OF THE ACETALANTHRAQUINONES ON V79 CELL VIABILITY

The effect of the acetalanthraquinones and ametantrone on V79 cell viability following drug incubation periods of 4 and 24 h is shown in table 5.3. EC_{50} represents the concentration of drug required to reduce the viability of the cell population by 50%. Figures 5.17 to 5.19 show the corresponding cytotoxicity curves for the acetalanthraquinones. Both exposure times produced the same order of potency (ametantrone >YCG9 >YCG8 >YCG7), the longer incubation period enhancing the cytotoxicity of all four compounds

Table 5.3: The effect of the acetalanthraquinones and ametantrone on V79 cell survival following drug incubation periods of 4 and 24 hours

DRUG	EC50 4 h (μM)	EC50 24 h (μM)
ametantrone	2.9 ± 0.6	1.6 ± 0.01
YCG7	150.6 ± 6.9	51.5 ± 5.0
YCG8	35.9 ± 2.5	5.9 ± 0.8
YCG9	13.6 ± 4.0	1.9 ± 0.1

EC_{50} represents the concentration of drug required to reduce the viability of the cell population by 50%. Values represent the mean ± sd for three separate experiments performed in triplicate (acetalanthraquinones) and the mean ± sd for one experiment performed in triplicate (ametantrone).

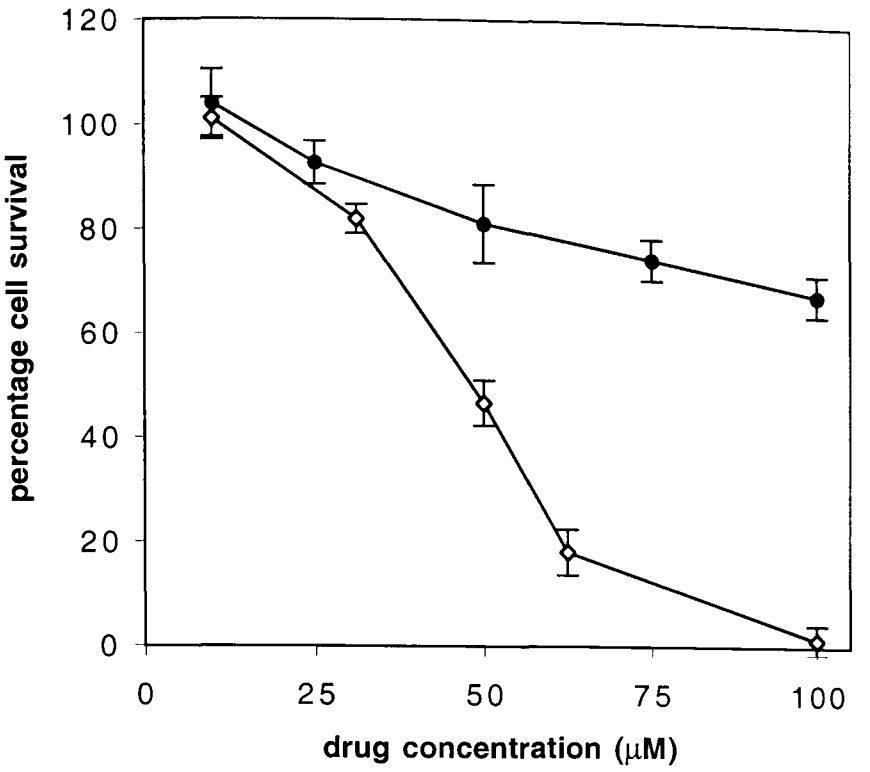


Figure 5.17: Effect of YCG7 on V79 cell survival following drug incubation periods of 4 (•) and 24 (◊) hours. The values shown represent the mean ± sd of one of three separate experiments.

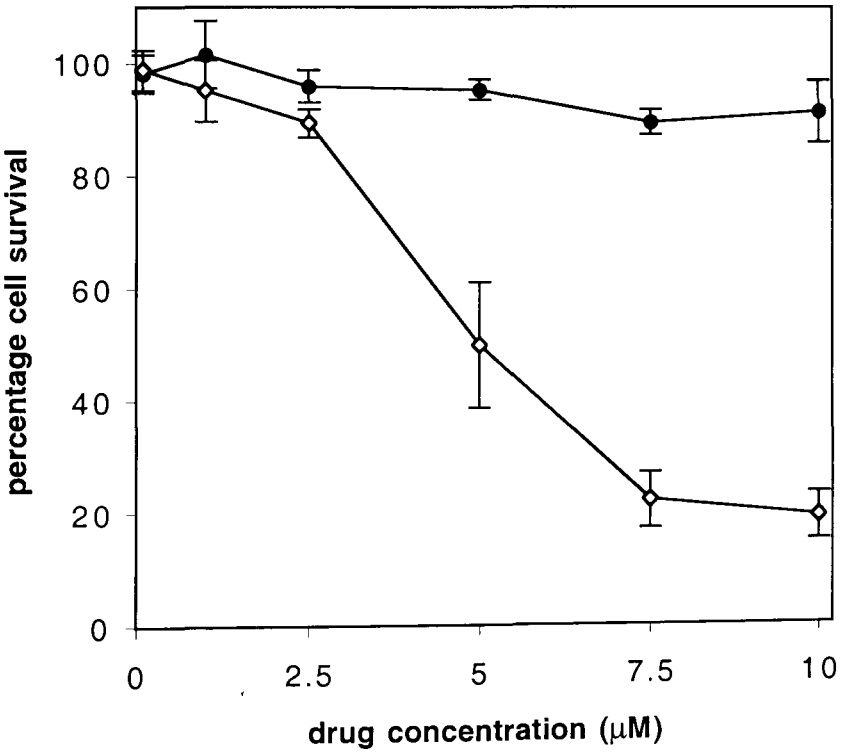


Figure 5.18: Effect of YCG8 on V79 cell survival following drug incubation periods of 4 (•) and 24 (◊) hours. The values shown represent the mean ± sd of one of three separate experiments.

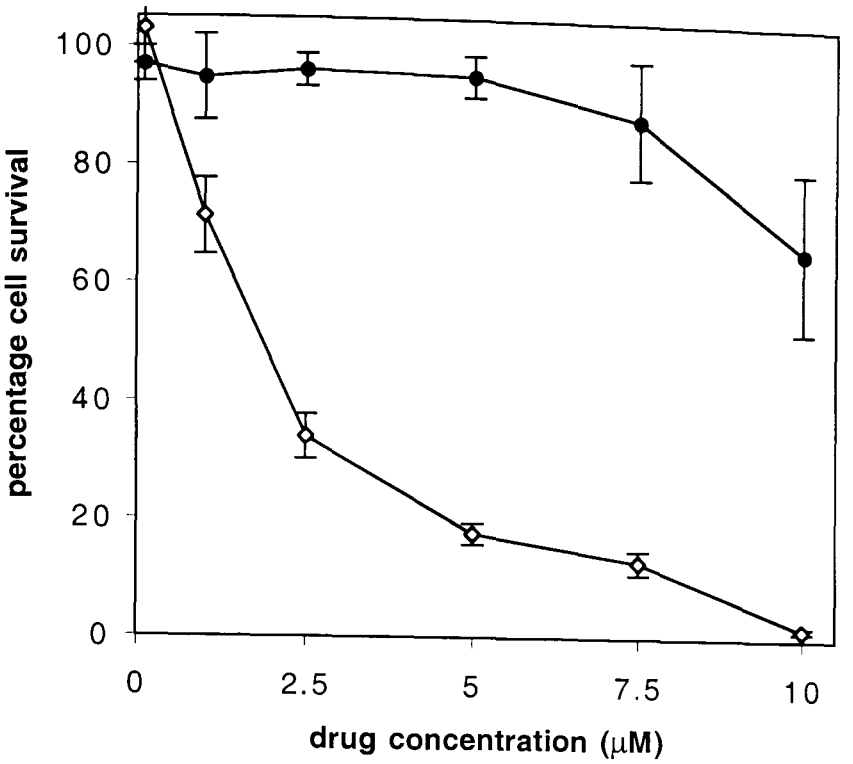


Figure 5.19: Effect of YCG9 on V79 cell survival following drug incubation periods of 4 (•) and 24 (◊) hours. The values shown represent the mean ± sd of one of three separate experiments.

On the basis that the difference in activity between ametantrone and the YCG compounds was more marked with a 4 h than a 24 h drug exposure, 4 h was chosen for subsequent cytotoxicity studies as it was thought that this would also enhance any differences in activity between the acetalanthraquinones and their hydrolysed and metabolized products. As V79 CHL cells are devoid of endogenous cytochrome P-450 monooxygenase activity (Keyes *et al*, 1984), the enzyme believed to be responsible for *O*-demethylation of the acetalanthraquinones, it was necessary to hydrolyse/metabolize the drugs exogenously prior to incubation with cells (see sections 4.2.2 and 4.2.3 for experimental detail).

5.3.2.3 THE EFFECT OF HYDROLYSED ACETALANTHRAQUINONES ON V79 CELL VIABILITY

The effect of the acetalanthraquinones and their hydrolysed products on V79 cell viability is shown in table 5.4 (with serum) and table 5.5 (without serum). Figures 5.20 to 5.22 show corresponding cytotoxicity curves for each drug (with serum). Cytotoxic potentiation, CP corresponds to the ratio of EC_{50} values obtained for the hydrolysed:unhydrolysed drug.

Table 5.4: The effect of the acetalanthraquinones and their hydrolysed products on V79 cell survival in the presence of foetal calf serum

DRUG	EC_{50} (μM) -hydrolysis	EC_{50} (μM) +hydrolysis	CP
YCG7	150.6 ± 6.9	10.6 ± 3.1*	14.3
YCG8	35.9 ± 2.5	2.5 ± 0.6*	14.6
YCG9	13.6 ± 4.0	6.1 ± 0.2	2.3

All drugs were incubated with cells for 4 h. EC_{50} represents the concentration of drug required to reduce the viability of the cell population by 50%. CP (cytotoxic potentiation) corresponds to the ratio of EC_{50} values obtained for the hydrolysed: unhydrolysed drug. Values represent the mean ± sd for three separate experiments performed in triplicate. * indicates that the results were significantly different to the corresponding values obtained in the absence of FCS at $p = 0.01$ (see table 5.5).

Following hydrolysis, an increase in cytotoxicity was observed for all three compounds. This was more marked for YCG7 and YCG8 than for YCG9 and was apparent when cells were incubated either with or without foetal calf serum. However,

the cytotoxicity of the acetalanthraquinone products was greater when the drugs were incubated with serum than without (14.3-fold compared to 2.7-fold greater than the parent compound for YCG7 and 14.6-fold compared to 4.6-fold greater for YCG8). This was far less evident for YCG9 (2.3-fold compared to 2.0-fold). Using the T-test (Miller & Miller, 1993), the EC_{50} values obtained for the hydrolysis products of YCG7 and YCG8 with FCS were found to be significantly different to those obtained without FCS, at a confidence level of 99% ($p = 0.01$). In contrast, the cytotoxicity of the acetalanthraquinones *per se* was not affected by serum and that a different pattern of potency was observed for the hydrolysed products (YCG8>YCG9>YCG7) than for the acetalanthraquinones (YCG9>YCG8>YCG7). Cell viability was unaffected by HCl/NaOH at the concentrations used.

Table 5.5: The effect of the acetalanthraquinones and their hydrolysed products on V79 cell survival in the absence of foetal calf serum

DRUG	EC_{50} (μM) -hydrolysis	EC_{50} (μM) +hydrolysis	CP
YCG7	153.7 ± 6.4	56.9 ± 3.4*	2.7
YCG8	31.9 ± 3.7	7.0 ± 1.2*	4.6
YCG9	14.0 ± 0.7	7.0 ± 1.2	2.0

All drugs were incubated with cells for 4 h. EC_{50} represents the concentration of drug required to reduce the viability of the cell population by 50%. CP (cytotoxic potentiation) corresponds to the ratio of EC_{50} values obtained for the hydrolysed: unhydrolysed drug. Values represent the mean ± sd for three separate experiments performed in triplicate. * indicates that the results were significantly different to the corresponding values obtained in the presence of FCS at $p = 0.01$ (see table 5.4).

The values shown in tables 5.4 and 5.5 and figures 5.20 to 5.22 represent the maximum increase in activity for each compound (corresponding to 12 h hydrolysis for YCG7 and 9 h for YCG8 and YCG9). EC_{50} values for YCG8 incubated with and without serum after 4.5 h hydrolysis were 6.9 ± 0.8 and 10.7 ± 1.4 respectively and after 6 h hydrolysis were 5.5 ± 1.2 and 7.2 ± 0.4 respectively. EC_{50} values for YCG9 incubated with and without serum after 6 h hydrolysis were 7.0 ± 0.6 and 7.8 ±0.8 respectively. Thus product formation coincided with an increase in cytotoxicity.

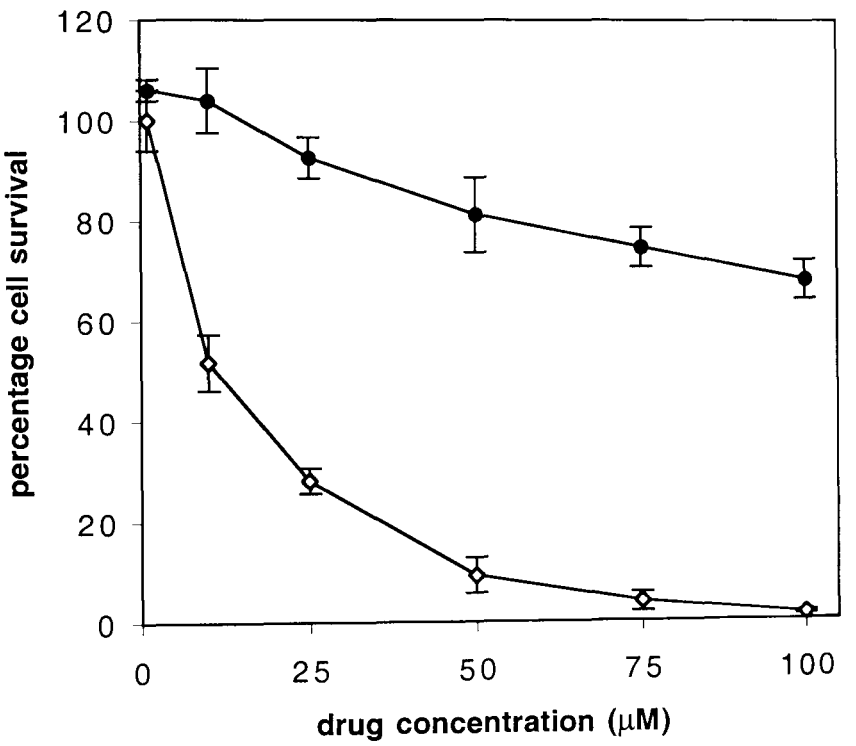


Figure 5.20: Effect of YCG7 on V79 cell survival before (•) and after (◊) drug hydrolysis. Values shown represent the mean ± sd of one of three separate experiments.

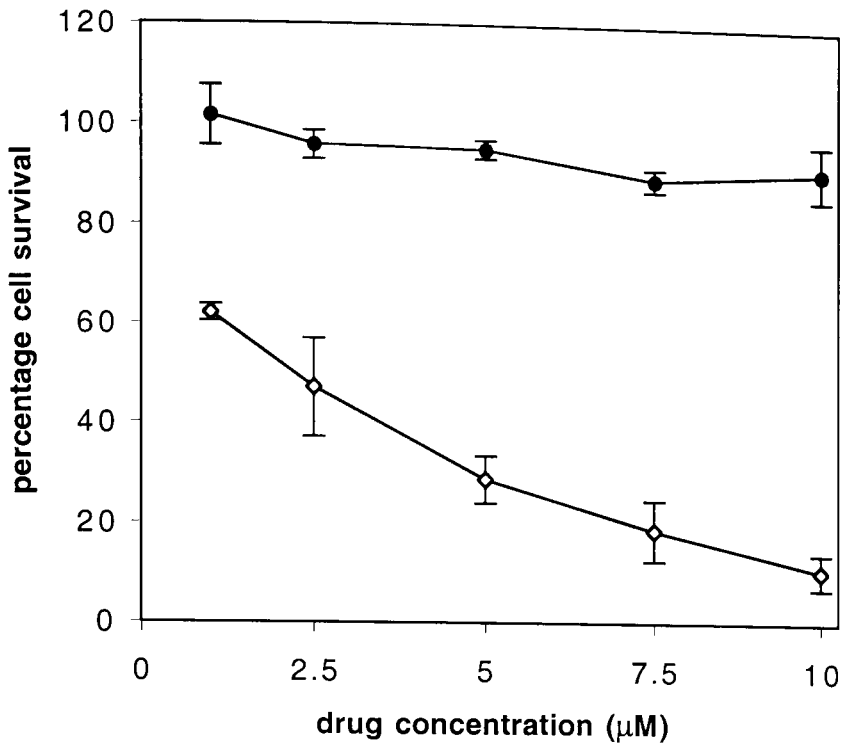


Figure 5.21: Effect of YCG8 on V79 cell survival before (•) and after (◊) drug hydrolysis. Values shown represent the mean ± sd of one of three separate experiments.

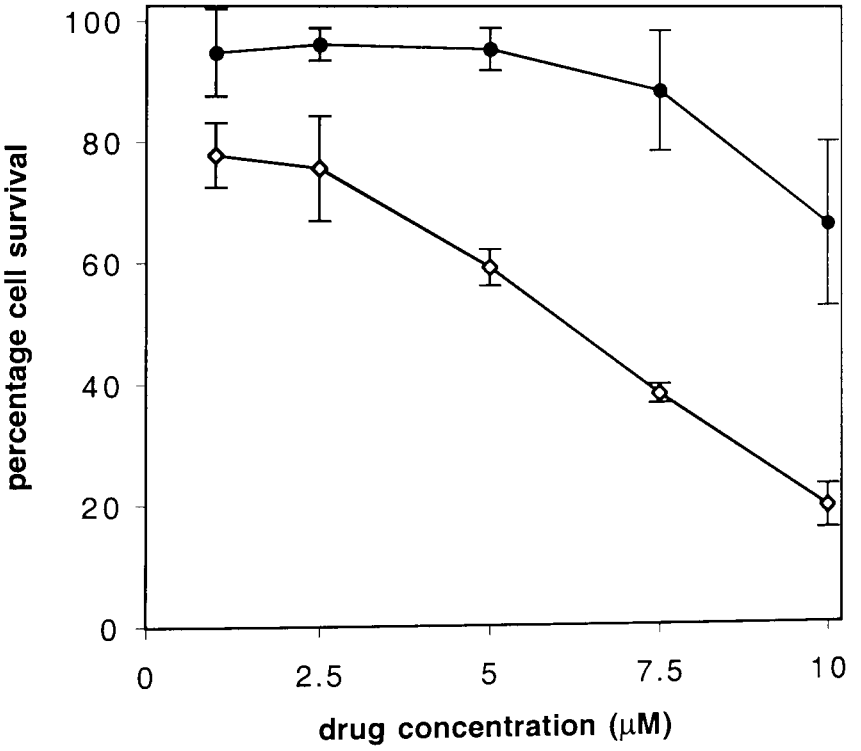


Figure 5.22: Effect of YCG9 on V79 cell survival before (•) and after (◊) drug hydrolysis. Values shown represent the mean ± sd of one of three separate experiments.

5.3.2.4 THE EFFECT OF METABOLIZED ACETALANTHRAQUINONES ON V79 CELL VIABILITY

The effect of the acetalanthraquinones and their metabolites on V79 cell viability after a 4 h drug incubation period are shown in table 5.6. The concentration of metabolites added to cells, determined by HPLC analysis, was essentially the same for YCG7, YCG8 and YCG9 (50± 5%), so the cytotoxicity results were directly comparable. The metabolites of YCG7 and YCG8 were considerably more cytotoxic than their respective acetalanthraquinones (4.4-fold for both compounds). In contrast, a smaller increase in cytotoxicity was seen following the metabolism of YCG9 (2.7-fold). For all three compounds, using the T-test (Miller & Miller, 1993), the EC_{50} values obtained after metabolism were found to be significantly different to those obtained before metabolism, at a confidence level of 99% ($p = 0.01$). In the absence of drug, the microsomal suspension had no effect on V79 cell viability. Similarly, 2% penicillin/ streptomycin had no effect on the viability of cells.

Table 5.6: The effect of the acetalanthraquinones and their metabolites on V79 cell survival

DRUG	EC_{50} (μM) -metabolism	EC_{50} (μM) +metabolism	CP
YCG7	25.4 ± 2.5	5.8 ± 3.3*	4.4
YCG8	12.8 ± 1.9	2.9 ± 1.7*	4.4
YCG9	18.3 ± 2.6	6.9 ± 2.1*	2.7

Drugs were incubated with cells for 4 h. EC_{50} represents the concentration of drug required to reduce the viability of the cell population by 50%. CP (cytotoxic potentiation) corresponds to the ratio of EC_{50} values obtained for metabolised: unmetabolised drug. Values represent the mean ± sd for three separate experiments. * indicates that the EC_{50} values were significantly different from those obtained for unmetabolised drug ($p = 0.01$).

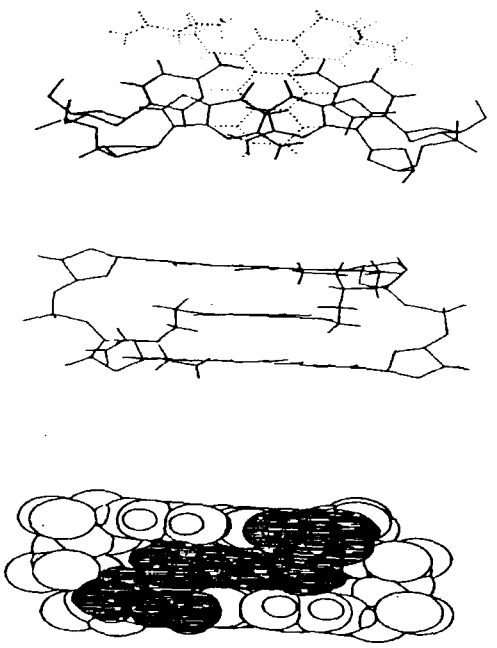
5.4 DISCUSSION

5.4.1 DNA BINDING STUDIES

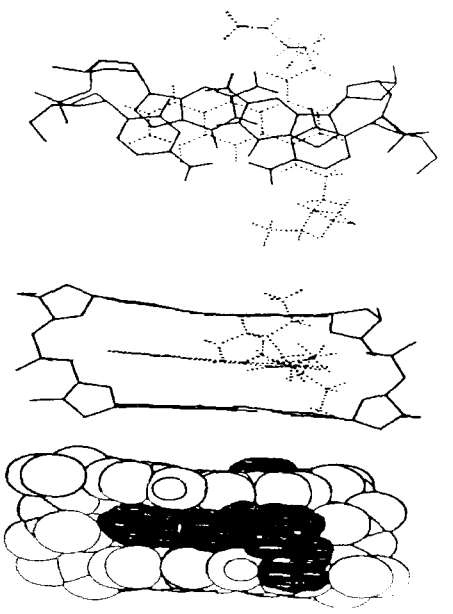
Overall, the acetalanthraquinones were weaker DNA intercalators than the structurally related ametantrone. The poor DNA binding affinity of YCG7, reflected in its very low ΔTm and small bathochromic and hypochromic shifts in the presence of DNA, was almost certainly due to the reduced ability of mono-substituted compounds to stabilize the intercalation complex by electrostatic attraction between the alkylamino side chain and the DNA phosphate backbone. Because mono-substituted compounds contain only one alkylamino side chain, they participate in less electrostatic binding with the DNA phosphate backbone, a process which usually stabilizes intercalation. In addition, the fact that there is only one side chain means that dissociation of the drug from the double helix is relatively unhindered. The results are supported by previous studies demonstrating that mono-substituted alkylaminoanthraquinones possess only weak intercalative properties (Islam *et al*, 1985) and dissociate more rapidly from DNA than bis-substituted compounds (Gandecha *et al*, 1985).

For YCG8 and YCG9, the bathochromic and hypochromic shifts at both 0.05M and 0.5M NaCl, and moderate increases in Tm, all indicated that these compounds were better DNA intercalators than YCG7. However, the values obtained were still lower than may be expected for bis-substituted anthraquinones (see sections 2.3.2.2 and 2.3.2.4). Two factors may contribute to the lower affinity of the acetalanthraquinones for DNA compared to ametantrone. Firstly, the bulky nature of the methoxy groups of the side chains may influence intercalation. This was also the case for AQ11 and AQ12, which each contain four hydroxyl groups in their alkylamino side chains (see section 2.3.2). Secondly, unlike the OH groups of the ametantrone side chains, the methoxy groups are not capable of hydrogen bonding with the nitrogens of the DNA base pairs and the DNA phosphate oxygens (Pohle *et al*, 1990), which would help to stabilize the intercalated complex.

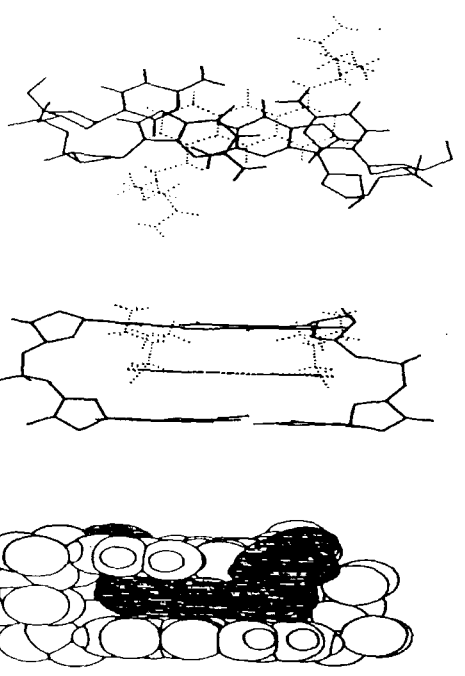
It can be concluded that the 1- and 1,5- substitution patterns of YCG7 and YCG9 were responsible for the absence of isosbestic points for these compounds. DNA binding studies performed in chapter 2 revealed a similar absence of isosbestic properties for the 1,5-bis-substituted compound AQ15 (see figures 2.5 and 2.6, section 2.3.2.2). The difference in the nature of DNA binding of YCG9 compared to YCG8 probably relates to their different modes of intercalation. Computer graphic modelling predicts two possible intercalative binding configurations for 1,4-substituted alkylaminoanthraquinones (figure 5.23), with the chromophore either perpendicular to the base pair axis with both side chains in the major groove or with the chromophore parallel to the base pair axis with the 1,4 bis alkylamino side chains lying in each groove 'straddling' the intercalation site. (Islam *et al*, 1985). High field NMR studies on oligonucleotide/mitoxantrone complexes (Lown & Hanstock, 1985), drug dissociation kinetics of DNA-drug complexes (Gandecha *et al*, 1985) and thermodynamic studies (Bell *et al*, 1989) all indicate that 1,4-bis-substituted compounds such as mitoxantrone favour the perpendicular mode of intercalation with their alkylamino side chains resting in the major groove. In contrast, 1,5-bis-substituted derivatives can enter via the major groove and straddle the intercalation site (Islam *et al*, 1985), but unlike 1,4 derivatives, they cannot intercalate in a perpendicular orientation. In order for straddling to be achieved, DNA breathing (transient base pair unstacking) must occur to allow the association of the 1,5 compound and the DNA receptor site. This enables a more stable complex to be formed, reflected in the slower dissociation rate of 1,5 derivatives from DNA (Bell *et al*, 1989). YCG9, being a 1,5 substituted compound, would be able to straddle the DNA helix, whereas the 1,4 derivative would probably favour perpendicular mode of binding.



A



B



C

Figure 5.23: Computer graphics modelling illustrating DNA intercalation of 1,4- and 1,5-bis-substituted alkylaminoanthraquinones. (A) shows the 1,4- major groove binding configuration, (B) shows 'straddling' of the intercalation site by 1,4- derivatives and (C) shows 'straddling' of the intercalation site by 1,5- derivatives. Reproduced from Islam *et al.* 1985.

In order to carry out DNA binding studies on the acetalanthraquinone products, the parent compounds were hydrolyzed for 12 h (YCG7) and 9 h (YCG8 and YCG9) as these times produced the greatest increase in cytotoxicity compared with the parent compounds (see section 5.3.2.3). Hydrolysis rather than metabolism was chosen to convert the acetalanthraquinones to their products so that potential interference from microsomal proteins could be avoided.

There was no apparent increase in DNA intercalation after hydrolysis, which is probably to be expected. Even if the acetalanthraquinones were converted to their corresponding aldehydes, they would be unlikely to form stable Schiff's bases with nucleic acids in the prevailing aqueous environment.

5.4.2 CYTOTOXICITY STUDIES

Ametantrone and the acetalanthraquinones were all found to inhibit the growth of V79 cells. However, ametantrone was considerably more potent than the other three compounds. This could be due to enhanced stabilization of DNA-topoisomerase II cleavable complexes by ametantrone, which contains 2'-(hydroxyethylamino)-ethylamino side chains (see section 2.4.1) and/or increased DNA binding affinity (see section 5.4.1). The lower DNA binding affinity of the acetalanthraquinones may also explain why extending the drug incubation period from 4 to 24 hours greatly enhanced their potency, because being less DNA affinic than ametantrone, they would require more time to accumulate in the nucleus.

The increase in cytotoxicity of YCG9 compared with YCG8 may be related its ability to 'straddle' the DNA helix, as discussed earlier (see section 5.4.1), and is consistent with a previous study in which 1,5-bis-substituted anthraquinones were more active than their 1,4-bis-substituted analogues (Islam *et al*, 1985). In support of this, the N-oxide of AQ5, the 1,5-substitued analogue of AQ4, has been shown to bind to DNA and is cytotoxic (Professor Laurence Patterson, personal communication). In addition, the different pharmacophores of 1,4- and 1,5-substituted compounds means they may stack differently within the DNA-topoisomerase II cleavage site (Capranico *et al*, 1997; see figure 1.11, section 1.3.2.4). The fact that YCG7 was considerably less potent than the bis-substituted compounds, can be partly attributed to its weak DNA binding affinity. Indeed, it is well known that higher doses of mono-substituted anthraquinones are required to achieve activity (Murdock *et al*, 1979; Zee-Cheng & Cheng, 1979).

After hydrolysis, the acetalanthraquinone products were still relatively non-potent compared with those of some other types of activated compounds, including the morpholino anthracyclines. Nonetheless, the hydrolysed products of YCG7, YCG8, and to a much lesser extent YCG9, were far more potent than the parent compounds. This may be accounted for by an increase in DNA binding, or possibly relates to increased interaction with other drug targets. In support of this, in a study by Holmes (1995), the

products of YCG7 and YCG8 products derived from metabolic activation demonstrated significant inhibition of topoisomerase II, whereas their respective parent compounds were inactive. In contrast, YCG9 inhibited topoisomerase II without being metabolized. Thus, enhanced interaction of YCG7 and YCG8 hydrolysis products with topoisomerase II may at least partly account for their enhanced potency in this study and may explain the smaller increase in cytotoxicity of the YCG9 hydrolysis products.

The cytotoxicity studies produced some interesting observations, and possible explanations for these have been given below. However, it is recognised that while the findings are true for the V79 cell line, their applicability across a number of cell lines was not established, and further studies should be carried out in order to establish their significance.

One rather unexpected discovery was the influence of foetal calf serum on the activity of the acetalanthraquinone products. Initially, serum was excluded from the culture medium during drug incubation with the aim of preventing covalent binding of aldehyde drug products to serum proteins, instead of their intended nuclear target(s). However, rather unexpectedly, incubation of the acetalanthraquinone products with serum actually enhanced their potency. It can be postulated that serum proteins were facilitating the transport of products into the cells in a similar manner to macromolecules such as BSA and dextran, which have been used to carry drug molecules into cells via endocytosis (Trouet *et al*, 1982, Shih *et al*, 1991). Serum-mediated drug uptake would probably involve formation of reversible Schiff's bases between the aldehydes and nucleophilic sites within the proteins. Once inside the cell, other nucleophiles may facilitate the mobilization of aldehyde products towards the nucleus (see later). Due to their reduced ability to react with nucleophiles, facilitated drug transportation of the acetalanthraquinones by this method would be far less feasible.

The presence of serum during drug incubation may further enhance the potency of acetalanthraquinone products in the V79 cells by stimulating the production of intracellular enzymes. In serum-starved human skin fibroblast cells, topoisomerase II levels were increased from 10^4 to 10^6 copies per cell by the addition of serum to the growth medium, corresponding to an increase in topoisomerase II mRNA expression of more than 70-fold (Hsiang *et al*, 1988). Although topoisomerase II in transformed cells is generally less sensitive to changes in serum levels than in normal cells (Holley, 1975; Hsiang *et al*, 1988), there is evidence that actively cycling V79 cells contain elevated levels of this enzyme (Dillehay *et al*, 1987) and studies have generally indicated a positive correlation between enzyme content and sensitivity to topoisomerase II inhibitors in cultured cells (Sullivan *et al*, 1987; Davies *et al*, 1988; Deffie *et al*, 1989; Kasahara *et al*, 1992). Thus, assuming that topoisomerase II was a target of the acetalanthraquinone metabolites, their effect would be enhanced by serum growth factors. Topoisomerase II α may be a preferential target for these compounds, as high levels of this isoform are associated with rapidly proliferating cells, topoisomerase II β being the predominant isoform expressed in quiescent cells (Woessner *et al*, 1991; Kimura *et al*, 1994).

Since V79 CHL cells are devoid of endogenous cytochrome P-450 monooxygenase activity, it was necessary to generate the acetalanthraquinone metabolites exogenously. However, the advantage of this was that it allowed the effect of the products on V79 cells to be determined without the potential complication of intracellular metabolite formation. Acetalanthraquinones incubated in the presence of NADPH-fortified mouse liver microsomes all showed increased activity compared with the parent compounds, particularly YCG7 and YCG8. On this basis, a number of speculations can be made about the behaviour of the acetalanthraquinone metabolites. However, it must be stressed that these arguments are conjectural, and further investigations are required in order to determine the nature of the metabolites (see

section 6.2).

It can be argued that the increased cytotoxicity of the YCG7 and YCG8 metabolites (and hydrolysis products) compared with the acetalanthraquinones in V79 cells was due to the formation of covalently reactive aldehydes. In all probability, on entering the cells, aldehydes would be subject to attack by molecules containing nucleophilic groups, for example, the α-amino groups of amino acids, resulting in the formation of Schiff's bases (imines) (Rawn, 1983; Fessenden & Fessenden, 1986). This involves the addition of the nucleophilic amine to the partially positively charged carbonyl carbon of the aldehyde, resulting in loss of a proton from the nitrogen and the gain of a proton by the oxygen (figure 5.24). The resulting OH group is then further protonated and is subsequently eliminated as water. In aqueous environments this reaction is reversible, allowing continual formation and dissociation of the imine, so this process could aid the mobilization of the aldehyde metabolites through the cells.

Assuming the metabolites follow a similar intracellular distribution pattern to structurally related agents (see section 1.2.2), it seems likely that they would eventually accumulate in the nucleus and intercalate DNA, providing an opportunity for the drug side chains to form Schiff's bases with amino acids within the topoisomerase II protein. As the DNA-topoisomerase II active site is non-aqueous (Capranico *et al*, 1997) there is a strong possibility that the Schiff's bases formed therein would be irreversible. This could give rise to persistently trapped topoisomerase II cleavable complexes in the hydrophobic interior of the topoisomerase II-DNA active site.

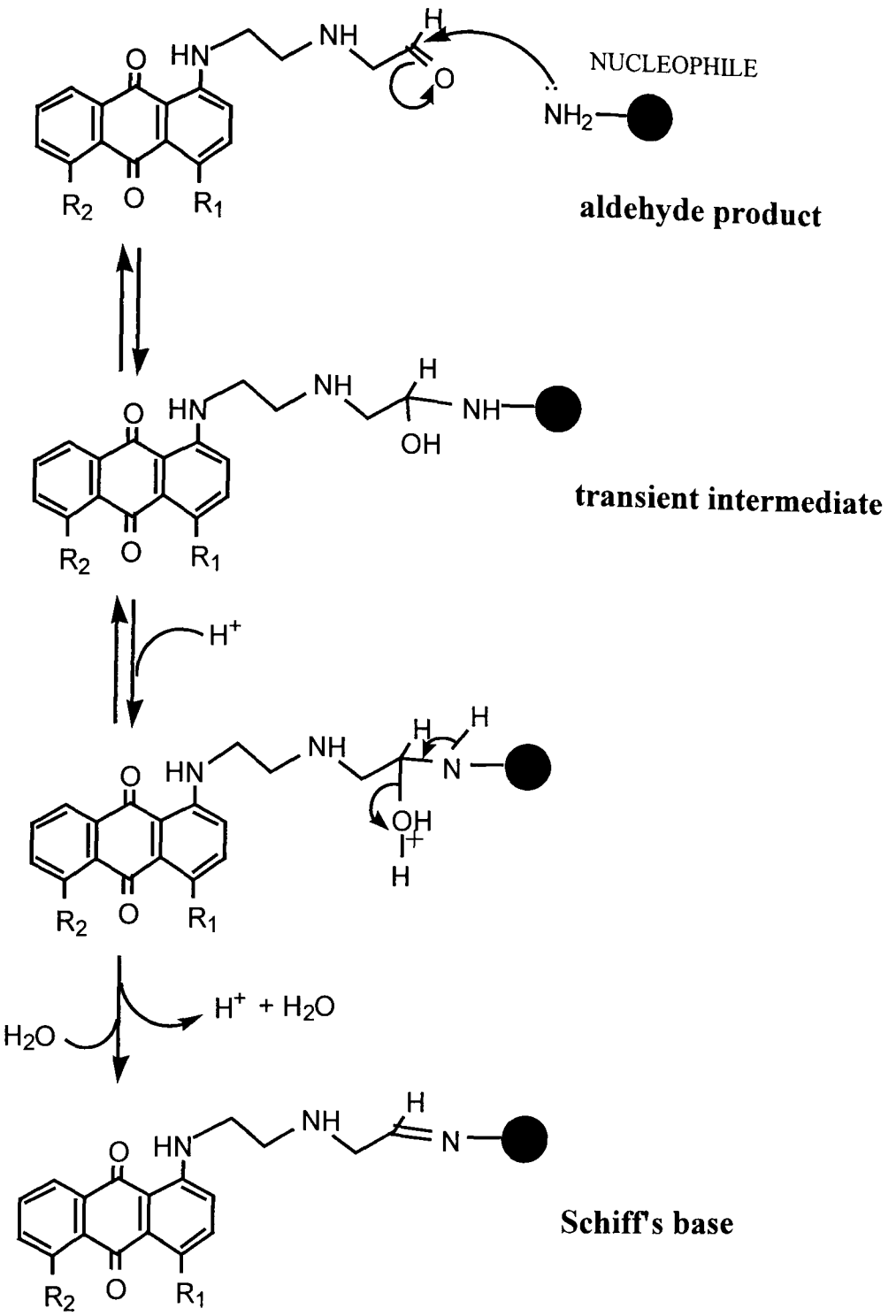


Figure 5.24: Proposed mechanism of Schiff's base formation between the aldehyde product of an acetalanthraquinone and nucleophilic centres of molecules such as topoisomerase II. YCG7: R1=R2=H; YCG8: R1=$NHCH_2CH_2NHCH_2CH(OCH_3)_2$, R2=H; YCG9: R1=H, R2= $NHCH_2CH_2NHCH_2CH(OCH_3)_2$.

Covalent binding has been implicated in the activity of several other antitumour agents. Amongst these are the doxorubicin analogues mdox and mmdox (see section 1.5.3). When mmdox is administered *in vivo*, an 80-fold increase in potency is observed compared with the parent drug (Ripamonti *et al*, 1992). *In vitro*, incubation of this compound with NADPH-fortified human or murine liver microsomes potentiates its cytotoxic potency 50-fold in the human ovarian carcinoma line ES-2 (Lau *et al*, 1994a). Cytotoxic potentiation is associated with the formation of activated metabolites, capable of forming DNA interstrand cross-links. Cytochrome P-450, principally the CYP3A subfamily, appears to play a major role in the biotransformation process (Lau *et al*, 1991, 1994a). Similar to the acetalanthraquinones, the active metabolite of mmdox is thought to be the *O*-demethyl derivative (Lau *et al*, 1991). DNA-drug adducts following metabolism have been detected by gel electrophoresis and HPLC but have not yet been identified (Graham *et al*, 1992). There is also strong evidence that the epipodophyllotoxin etoposide is activated by cytochrome P-450-mediated *O*-demethylation of the dimethoxyphenol ring. The *O*-demethylated product has a 10-fold increase in binding to calf thymus DNA (Van Maanen *et al*, 1987). It also induces inactivation of single-stranded and double-stranded ϕX174 DNA and displays enhanced cytotoxic activity in Aux-B and H35 cells (Van Maanen *et al*, 1985c, 1987). These findings support the hypothesis that cytotoxicity of the acetalanthraquinones may have been enhanced by metabolic activation to products which can cause DNA damage.

Because a small amount of drug remained bound to microsomal protein after metabolism, slightly less drug was added to cells than in the case of the control drug incubations. This almost certainly led to an underestimation of their cytotoxicity, which was probably compounded by the fact that the most highly protein affinic compounds were also likely to be the most potent. In addition, a mixture rather than isolated metabolite was added to cells. However, the EC_{50} values obtained were comparable in that the cells were incubated with the same proportion of acetalanthraquinone: metabolite (50:50) for each compound.

A discrepancy was seen in the activity of the parent compounds when comparing the hydrolysis and the metabolism studies. For YCG7 and YCG8, the non-metabolized controls were more active than the non-hydrolysed controls. This may have been due to the transfer of a residual amount of microsomal protein from the metabolic incubates into the V79 cells upon drug incubation, supplying exogenous cytochrome P-450 monooxygenase activity for drug metabolism. Nevertheless, the cytotoxic potentiation (CP) values after metabolism displayed a similar pattern to those obtained following hydrolysis, in the fact that CP for metabolized YCG7~YCG8 and hydrolysed YCG7~YCG8. No discrepancy was observed for YCG9, which showed comparable results in both systems.

In summary, the increase in cytotoxicity of the hydrolysed and metabolized products compared with the acetalanthraquinones YCG7 and YCG8 supports the concept that they were converted to their corresponding aldehydes. Increased drug interaction with DNA processing enzymes such as topoisomerase II via formation of persistent (Schiff's base) complexes between DNA/topoisomerase II and aldehyde products may have occurred. YCG9 was found to be relatively cytotoxic *per se*. This could be at least partly related to its mode of DNA binding, which is likely to involve spearing of the DNA. Cytotoxicity of the products was enhanced by the presence of serum during the drug incubation period, perhaps because of facilitated drug transport or up-regulation of intracellular topoisomerase II levels in conjunction with an increased rate of cell proliferation.

CHAPTER 6

6.1 CONCLUSIONS

DNA intercalating agents such as the anthraquinones and anthracyclines have a well-established role in the treatment of a wide variety of human cancers. However, due to their systemic and specific toxicities, the amount of drug that can be administered to patients is limited. The need to reduce the toxicity of therapeutically active antitumour agents has resulted in extensive research into the development of new compounds with improved activity and diminished side effects. One strategy has been to synthesize compounds which can be targeted specifically to their site of action in the form of prodrugs. These are agents that are chemically or metabolically activated *in vivo* to produce the pharmacologically active species. Recent advances in tumour selective activation of anticancer agents had led to a renewed interest in prodrugs. This has primarily been due to the emergence of new techniques such as ADEPT and GDEPT, and exploitation of the chronic hypoxia and low external pH that is prevalent in most solid tumours. In addition, cytochrome P-450s (and other enzymes) are known to be overexpressed in a whole range of malignancies, including those of the breast colon, and lung - the three main refractory killers (see section 1.5.1). Moreover, the discovery of the human tumour specific cytochrome P-450 isoform CYP1B opens up new possibilities for tumour specific development of metabolically activated compounds (Murray *et al*, 1997).

The prodrug AQ4N, developed and synthesized at De Montfort University (Patterson *et al*, 1992; Smith *et al*, 1997a, 1997b), will be undergoing phase I clinical trials in the year 2000 for the treatment of non-small cell lung cancer. The intrinsic advantage of AQ4N over radiosensitizing agents and some bioreductive agents is that until the drug is reduced to AQ4, it is almost completely inactive. The present study clearly indicated that the low toxicity of AQ4N is due to modification of the terminal nitrogen of the cationic alkylamino side chain to form an electrically neutral N-oxide

functionality. This prevents stabilization of intercalative binding normally associated with cationic alkylaminoanthraquinones, which in turn prevents topoisomerase II inhibition. Inability to achieve stable intercalation is attributable to a reduction in the electrostatic attraction between the amino group of side chains of drug and the negatively charged PO_4^- groups of DNA backbone.

An insight into the lack of potency of AQ4N has been gained using flow cytometric and confocal imaging studies of AQ4 and AQ4N in intact human cells (Smith *et al*, 1997a). These studies revealed that although the N-oxide is able to enter the cell nucleus, it is only retained effectively in the cytoplasm, whereas AQ4 targets the nucleus to a high degree. It has been suggested that due to its high affinity for DNA, AQ4 should remain localized in tumour tissue, hopefully long enough to kill cells when they resume cycling upon re-oxygenation. Similar observations have been made with respect to the cellular distribution of the mono-N-oxide AQ6N compared with its parent compound AQ6 (Smith *et al*, 1997a; see sections 2.3 and 2.4). However, being a mono-N-oxide, AQ6N retains some activity (Smith *et al*, 1997b) and is thus less suitable as a selectively activated bioreductive drug than AQ4N.

In contrast to the N-oxide functionality, the presence of the 2'-(hydroxyethylamino)ethylamino side chains of mitoxantrone was related to enhanced topoisomerase II inhibition and DNA binding activity. This was possibly due to oxidation of the side chains to produce aldehyde intermediates, which may increase the longevity of the topoisomerase II-DNA cleavable complex by Schiff's base formation with the enzyme. Given these observations, a series of acetalanthraquinones were developed, possessing dimethoxy groups in place of the alcohol groups of mitoxantrone. These compounds were designed on the basis that they could be converted to their respective aldehyde derivatives. It was proposed that these derivatives would be more active than the acetals due to the potential for Schiff's base formation with intracellular enzymes such as topoisomerase II.

Acid-catalysed hydrolysis and oxidative metabolism by NADPH-fortified mouse

liver microsomes both proved effective routes for the conversion of acetalanthraquinones. Enhanced potency was observed for both hydrolysed and metabolized products of YCG7 and YCG8 and an increase in drug interaction with topoisomerase II via formation of persistent (Schiff's base) complexes was proposed. YCG9 was found to be relatively cytotoxic *per se*, perhaps relating to its mode of DNA binding (Islam *et al,* 1985, Bell *et al,* 1989). Another study carried out by Holmes (1995) demonstrated the ability of activated YCG7 and YCG8 to inhibit topoisomerase II, whereas the corresponding acetals were inactive. This may indicate that non-activated YCG7 and YCG8 target enzymes other than topoisomerase II. Structurally related compounds have been shown to inhibit polymerases (Fox *et al*, 1996), ligases (Ciarrocchi *et al*, 1988) and helicases (Bachur *et al*, 1992). Further studies are required to establish the exact mechanisms of action of the acetalanthraquinones and their products (see section 6.2).

An increase in potency of the acetalanthraquinone products was observed in the presence of foetal calf serum. It is proposed that this was related to stimulation of topoisomerase II levels (probably topoisomerase IIα) coinciding with increased cell proliferation. Topoisomerase IIα levels have been shown to be low in serum-starved cells (Feister *et al*, 1997) and several studies have demonstrated that encouraging cells to increase their levels of topoisomerase II enhances their sensitivity to cytotoxic agents, including mitoxantrone and doxorubicin (Sullivan *et al*, 1987; Davies *et al*, 1988; Deffie *et al*, 1989; Kasahara *et al*, 1992). The association between growth factor stimulation of a cell population and enhanced expression of topoisomerase II offers the possibility of optimizing chemotherapy by manipulating the proliferative status of target cells. For example, stimulation of breast cancer cell lines using oestrogen results in the elevation of drug-induced protein-associated DNA strand breakage and a corresponding increase in cytotoxicity (Zwelling *et al*, 1983; Epstein & Smith, 1988). Similarly, in myeloid leukaemic cell lines expressing G-CSF receptor, the *in vitro* cytotoxicity of the topoisomerase II inhibitors can be significantly enhanced by

addition of exogenous G-CSF (Towatari *et al*, 1990). The results obtained in this study may signify the potential for development of acetal-containing compounds as antitumour agents in conjuction with growth factors. This would be particularly useful for the treatment of slow growing tumours, as cancer cells from patients containing low levels of topoisomerase II are often refractory to treatment (Potesmil *et al*, 1988).

The acetalanthraquinones are analogous to the morpholino compounds in the fact that they are cytotoxic, even without activation, and thus are not true prodrugs. However, preliminary studies indicate that the increased potency of methoxymorpholino doxorubicin means that at therapeutically effective doses, the cardiotoxicity associated with doxorubicin should be avoided (Sikic *et al*, 1985; Danesi *et al*, 1993). Similarly, the acetalanthraquinone products were significantly more cytotoxic than the parent compounds. However, unlike the morpholino anthracycline metabolites, they were probably not potent enough to be clinically effective. This may be partly due to the fact that the acetalanthraquinones contained a non-hydroxylated chromophore, which in all probability would reduce their activity substantially (see sections 1.2.2 and 1.3.2.2). In addition, assuming the aldehyde was present, and that it was the most active component, its cytotoxicity would have been masked by the fact that it was added to the V79 cells as part of a mixture of products.

It should be pointed out that the metabolism of the acetalanthraquinones in humans may differ from that in mice. Indeed, a large interspecies variability in mitoxantrone biotransformation has also been demonstrated both *in vitro* and *in vivo* (Avramis, 1982; Richard *et al*, 1989, 1991; Blanz *et al*, 1991a, 1991b). Clearly, the most accurate insight into the biotransformation of cytotoxic agents in a clinical setting is to be gained by working with human samples rather than those from animals. Thus, further studies are required to investigate the metabolism of acetalanthraquinones using human enzyme systems (see section 6.2).

Altered expression of drug metabolising enzymes in certain types of cancer cells compared with normal tissues is thought to be basis for the selectivity of the alkylating

agent cyclophosphamide against certain tumour types (section 1.5.2.2). Unfortunately, at present, there is a shortage of compounds like cyclophosphamide in clinical use. However, due to the increasing prevalence of cancer in modern society, there is an ever-growing demand for such compounds. Drugs such as mdox, mmdox, AQ4N and several others already show promise in this respect (reviewed by Denny, 1996). Hopefully, as our understanding of cancer cell enzymology increases, it should be possible to exploit the differences between normal- and cancer cells to design other tumour specific agents. It is conceivable that one of the discoveries made could lead to the use of acetal-containing compounds in prodrug therapy.

6.2 FUTURE WORK

Although the results obtained so far are encouraging, further studies are required to investigate the activity of the acetalanthraquinones using human enzyme systems. A useful approach would be to transfect human cytochrome P-450 monooxygenase enzymes into V79 cells. Fortuitously, this cell line is devoid of endogenous CYP activity, with the result that working with CYP-expressing V79 cells would be akin to using the purified enzyme. Successful transfer of several CYPs into the V79 cell line, including CYP1A1, CYP1A2 and the human tumour-specific isoform CYP1B1 has already been achieved (Doehmer & Schmalix, 1994; Schmalix *et al*, 1996; Luch *et al*, 1998). In addition, non-P450 enzymes, such as dehydrogenases or oxidases, may also be involved in the oxidation of the acetalanthraquinones (see Gibson & Skett, 1986; Beedham, 1997; section 1.5.1). Studies should also be carried out to investigate the metabolism of acetalanthraquinones in tissues where the expression of cytochrome P-450s and other drug-metabolizing enzymes is different from that in normal cells (see section 1.5.1).

A common feature of cancer cells is their ability to overproduce lactic acid (Baggetto, 1997; Dang *et al*, 1997). Extracellular accumulation of lactic acid means that the pH surrounding solid tumours is usually lower than normal, the precise acidity depending on the type of malignancy. Thus, by virtue of their instability in acidic environments, the acetalanthraquinones may be hydrolysed in the extracellular space, providing another opportunity for the exploitation of the unique properties of tumour cells. Although the cytosolic pH of tumour cells is not acidic, the pH of the lysosomal compartment is about pH 5.5 and several attempts have been made to target the lysosomal compartment using acid-labile proderivatives (Masquelier *et al*, 1980; Sat *et al*, 1999). This could provide another means of converting the acetalanthraquinones to their more active products. Although the acetalanthraquinones were stable to hydrolysis under conditions which mimic those of the lysosomal compartment (37°C, pH5.5), hydrolytic enzymes present in the lysosomes (Alberts *et al*, 1983) may lower the

activation energy required for hydrolysis, so it could occur at 37°C. Incubation of the acetalanthraquinones with isolated liver lysosomes may help to test the feasibility of targeting these compounds to the lysosomal compartment.

Further studies are required to achieve a greater insight into the mechanism(s) of action of the acetalanthraquinones. For example, the cleavable complexes formed by most topoisomerase II-targeting drugs are readily reversed if exposed to high salt concentrations (Tewey *et al,* 1984b) or heated to 65°C (Hsiang & Liu, 1989) prior to the addition of protein denaturants such as SDS. However, irreversible topoisomerase II cleavable complexes have been demonstrated for camptothecin analogues (Hertzberg *et al*, 1990) and the terpenoides, terpentecin and clerocidin (Kawada *et al*, 1991), which unlike the complexes formed by other topoisomerase II inhibitors, are stable to these treatments. The formation of irreversible cleavable complexes by the camptothecin analogue 10-bromoacetamidomethylcamptothecin is thought to be mediated by covalent linkage with the topoisomerase I-DNA adduct, preventing DNA ligation. Therefore, it would be interesting to investigate the stability of the cleavable complexes formed by the acetalanthraquinone products after activation. Formation of irreversible cleavable complexes by the acetalanthraquinone products would support the involvement of covalent linkage between aldehyde products and the topoisomerase II-DNA adduct as a mechanism of action. Moreover, it has not yet been determined to what extent each of the topoisomerase II isoforms is targeted by the acetalanthraquinones. This could be investigated using decatenation assay, or alternatively, a more recently developed assay employing *Sacharomyces cerevisiae* modified to express either human topoisomerase IIα or topoisomerase IIβ (Hammonds *et al*, 1998).

Both morpholino doxorubicin and methoxymorpholino doxorubicin show significant activity in *MDR1*-expressing doxorubicin-resistant cell lines (Streeter *et al*, 1986; Ripamonti *et al*, 1992; Lau *et al*, 1994a) and those displaying the at-MDR phenotype (Capranico *et al*, 1994, Mariani *et al*, 1994). Similar investigation into the activity of the acetalanthraquinones in mitoxantrone-resistant cell lines (e.g.the HL-60

human leukaemia cell line) is required. It would also be interesting to assess the activity of the acetalanthraquinones in V79 cells resistant to other topoisomerase inhibitors, such as the DC-3F/9-OH-E cell line (Khelifa *et al*, 1994). Resistance in this line is associated with a four- to five-fold reduction in topoisomerase II α and a ten-fold reduction in the ability of topoisomerase inhibitors to induce cleavable complex formation. The cytotoxicity of the acetalanthraquinone products, particularly those of YCG7 and YCG8, should also be investigated in cell lines such as the VP-16- and amsacrine-sensitive ataxia-telangiectasia cell line, which overexpresses topoisomerase II and shows elevated levels of drug-induced cleavable complexes (Smith & Makinson, 1989).

The active metabolite of methoxymorpholino doxorubicin is thought to be the *O*-demethyl derivative (Lau *et al*, 1991; see section 1.5.3.2). Although DNA-drug adducts following metabolism have been detected by gel electrophoresis and HPLC, the metabolite has not yet been identified (Graham *et al*, 1992). Similarly, as the acetalanthraquinone products generated in this study were not isolated, their identification was not possible. Isolation of the products would be more desirable than adding a mixture of products to cells, so that their individual potencies could be assessed. Use of a preparative HPLC system would allow relatively large quantities of products to be separated for this purpose. Structural identification could be performed by mass spectroscopy, followed by comparison of the mass spectrum fragmentation patterns with the HPLC retention times of the isolated products and synthetic reference samples. Once the structure of each component was known, it would also be interesting to study the fates of the acetalanthraquinones and their products using flow cytometric and confocal imaging techniques. Such techniques are already well established in studying the cellular distribution of the TNQ metabolite of mitoxantrone (Feofanov *et al*, 1997) and the N-oxides AQ4N and AQ6N (Smith *et al*, 1997a).

Despite the fact that the acetalanthraquinone products were considerably more active than their parent compounds, they were still poor cytotoxic agents compared to the metabolites generated by other activated drugs, for example, the morpholino

anthracyclines. Thus, there is clearly a need to produce acetal-containing compounds that will be sufficiently potent when activated to be effective in clinical setting. These should include compounds with hydroxylated chromphores and also with different chromophores. One option would be to attach the acetal side chains to the anthrapyrazole moiety, which appears to produce compounds that are less cardiotoxic than many of the anthraquinones and anthracyclines (see section 1.4.1.4). Moreover, it could be argued that YCG7 and YCG8 are topoisomerase II prodrugs because they do not inhibit the enzyme until metabolically activated (see section 5.4.2). By the same token, if drugs could be designed where the DNA binding activity of the parent compounds is reduced, this may lessen the side effects of the drug by lowering DNA binding in normal cells. Also, from a prodrug design point of view, a high DNA binding affinity is not desirable in the parent compound from which the prodrug is derived, because this considerably reduces drug diffusion to surrounding tumour cells.

Although the acetalanthraquinone products were disappointing in terms of their potency, the results are extremely promising in that they demonstrate that it is possible to produce acetal-containing compounds with the potential to be metabolically or chemically activated to agents with novel mechanisms of action. This concept can now be applied to produce a variety of other acetal-containing drugs, one or more of which may prove suitable for clinical use.

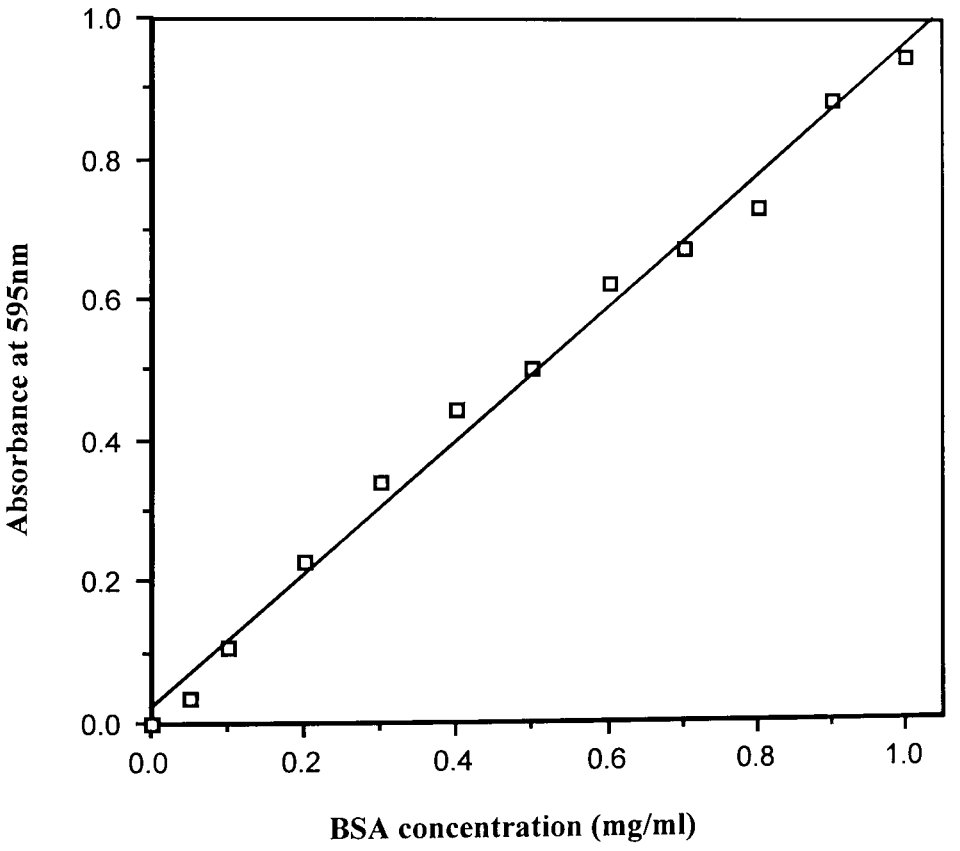
APPENDICES

A1: THE TRYPAN BLUE DYE EXCLUSION ASSAY

Trypan blue solution (100µl, at 0.4%) was added to 250µl of V79 cell suspension and left for 5 min. A Neubauer haemocytometer (total capacity 10^{-4} ml) was moistened around the edges of the counting chamber and a cover slip pressed into position over the chamber ensuring the formation of Newton's rings in the surrounding area. The counting chamber was filled with approximately 10µl of cell suspension. The proportion of lysed cells was ascertained by microscopic examination (only cells with disrupted membranes accumulate the trypan blue dye).

A2: THE BIORAD ASSAY

Bio-Rad reagent (a concentrate of Coomassie Brilliant Blue dye, methanol and phosphoric acid) was diluted five-fold with double distilled water and filtered. The reagent (5ml) was then added to bovine serum albumin (BSA; standard grade) solutions (0.1ml in 0.1M phosphate buffer, pH 7.4) ranging in concentration from 0-1.0 $mgml^{-1}$ and to samples of V79 nuclear extract and Balb C liver microsomes, which were also diluted in phosphate buffer, pH 7.4. The solutions were incubated at room temperature for 15 min and the absorbance was then measured at 595nm against a blank of double distilled water. A BSA calibration curve was constructed to determine the protein content (ml^{-1}) of the nuclear extract/microsomes.



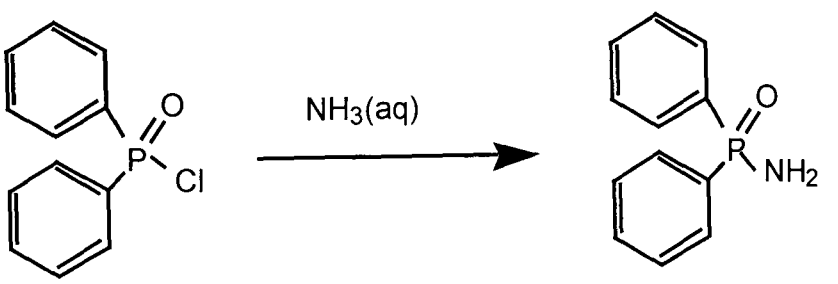
The microsomal protein concentration was 52.6 ± 2.1 $mgml^{-1}$.

The protein content of the V79 nuclear extract was 855 ± 35.7 μgml^{-1}.

A3: SYNTHESIS OF STARTING MATERIALS (Chapter 3)

A3.1 SYNTHESIS OF DIPHENYLPHOSPHINAMIDE

A 33% solution of aqueous ammonia (400ml) at 0°C, was added dropwise to 50g (0.2 mol) diphenylphosphinic chloride, with vigorous stirring, such that the temperature did not exceed 10°C. The product was filtered *in vacuo* and washed with water (4x60ml). The yield was 26.5g (61%).

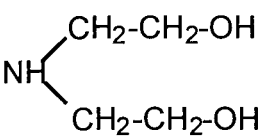


diphenylphosphinic chloride

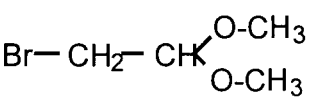
diphenylphosphinamide

A3.2 SYNTHESIS OF *N*-[2-(2'-METHOXYMORPHOLINYL)]ETHANOL

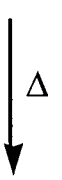
Bromoacetaldehyde dimethylacetal 100g (0.5 mol) was added to 93g (0.75 mol) diethanolamine over 1 hr at 60-70°C, then heated to 100-110°C for 2 to 4 hr. until the starting material was consumed (followed by thin layer chromatography in CH_2Cl_2/MeOH 9:1). The resulting mixture was distilled *in vacuo* at 2mmHg. collecting the distillate between 108 and 110°C. The yield was 53.1g (66%).

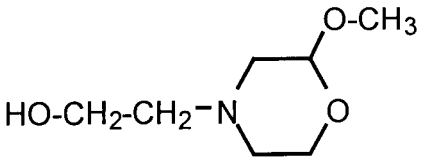


di(2-hydroxyethyl-amine)



Bromoacetaldehyde dimethylacetal

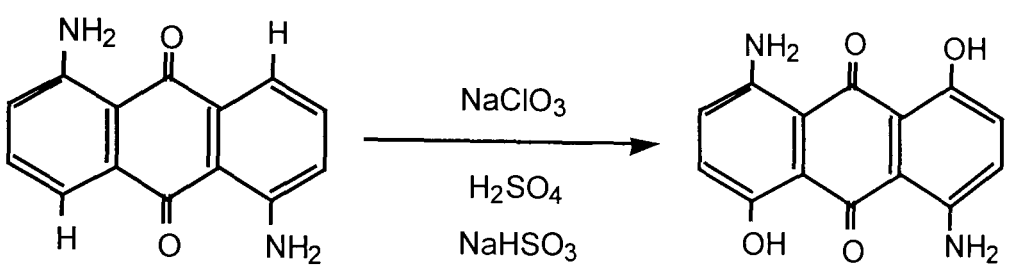




***N*-[2-(2'-methoxymorpholinyl)]ethanol**

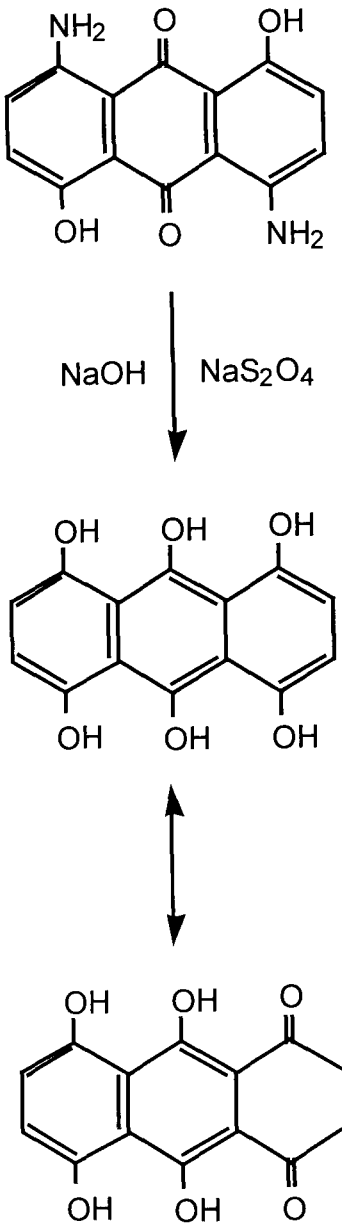
A3.3 SYNTHESIS OF 2,3-DIHYDRO-1,4,5,8-TETRAHYDROXY-ANTHRACENE-9,10-DIONE

Step 1: sodium chlorate, 72g (0.067 mol) was added, over a 30 minute interval, to a solution of 1,5-diamino-anthracene-9,10-dione, 6g (0.025 mol) in conc. H_2SO_4 (100ml) on ice. The mixture was allowed to cool to room temperature, and stirring was continued for a further 3h, after which time the reaction mixture was poured into one litre of 1% sodium hydrogen sulfite. The resulting precipitate was removed by filtration *in vacuo* and washed with cold distilled water (2x500ml) and distilled water at 60°C (2x500ml). The solid was dried *in vacuo*. The yield was 5.9g (88%).



1,5-diamino-anthracene-9,10-dione **1,5-diamino-4,8-dihydroxy-anthracene-9,10-dione**

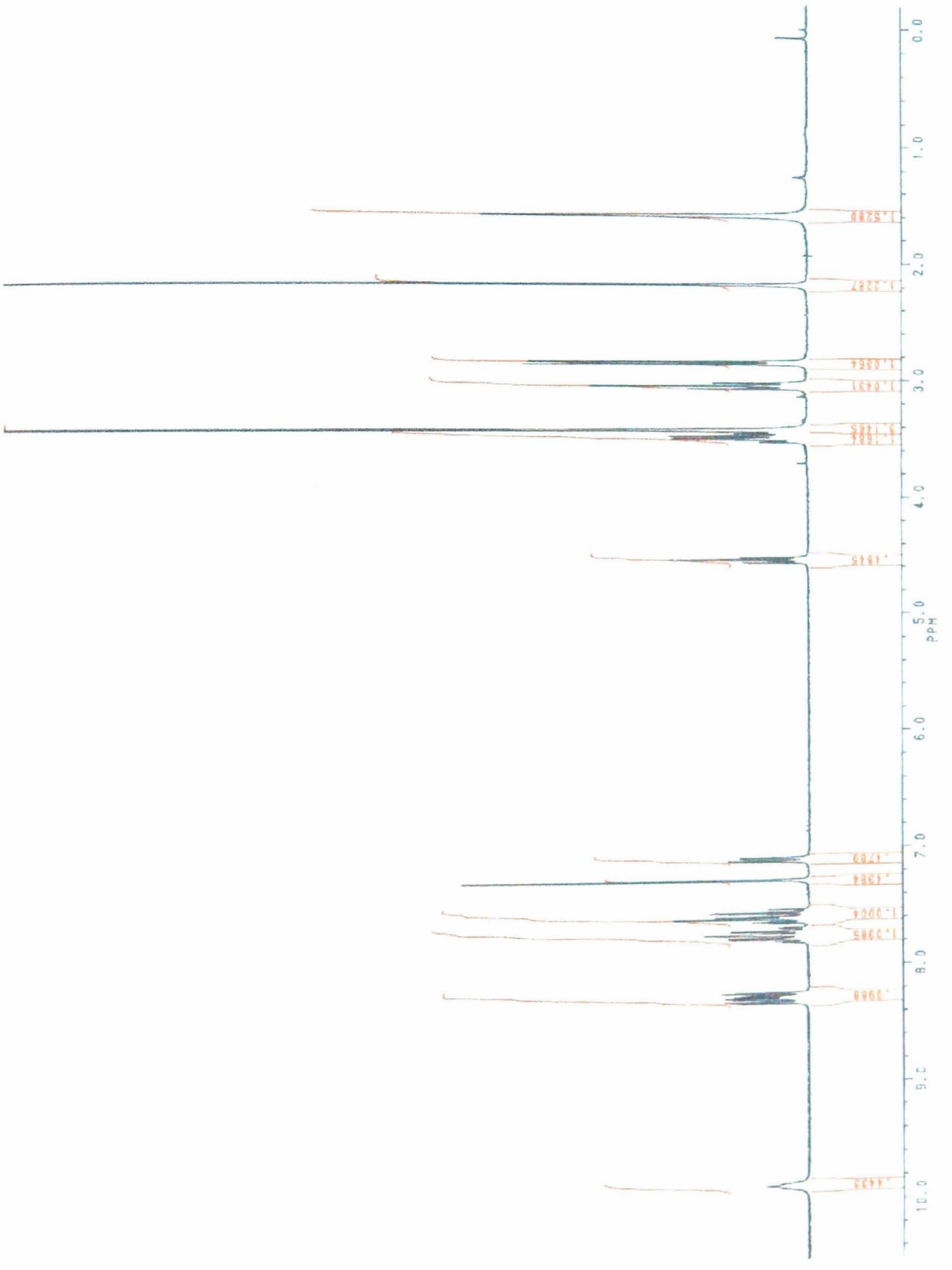
Step 2: to a solution of 1,5-diamino-4,8-dihydroxy-anthracene-9,10-dione, 4g (0.015mol) in 2.5M aqueous NaOH solution, under argon, at 50-60°C, was added 35g (0.2mol) sodium hydrosulfite, whilst increasing the temperature to 90-95°C. The mixture was heated until no starting material was present (followed by thin layer chromatography), then cooled to room temperature and acidified to pH 6.0 with 10M HCl. The resulting precipitate was filtered and washed with distilled water (4x100ml), and dried *in vacuo*. The yield was 2.9g (70%).

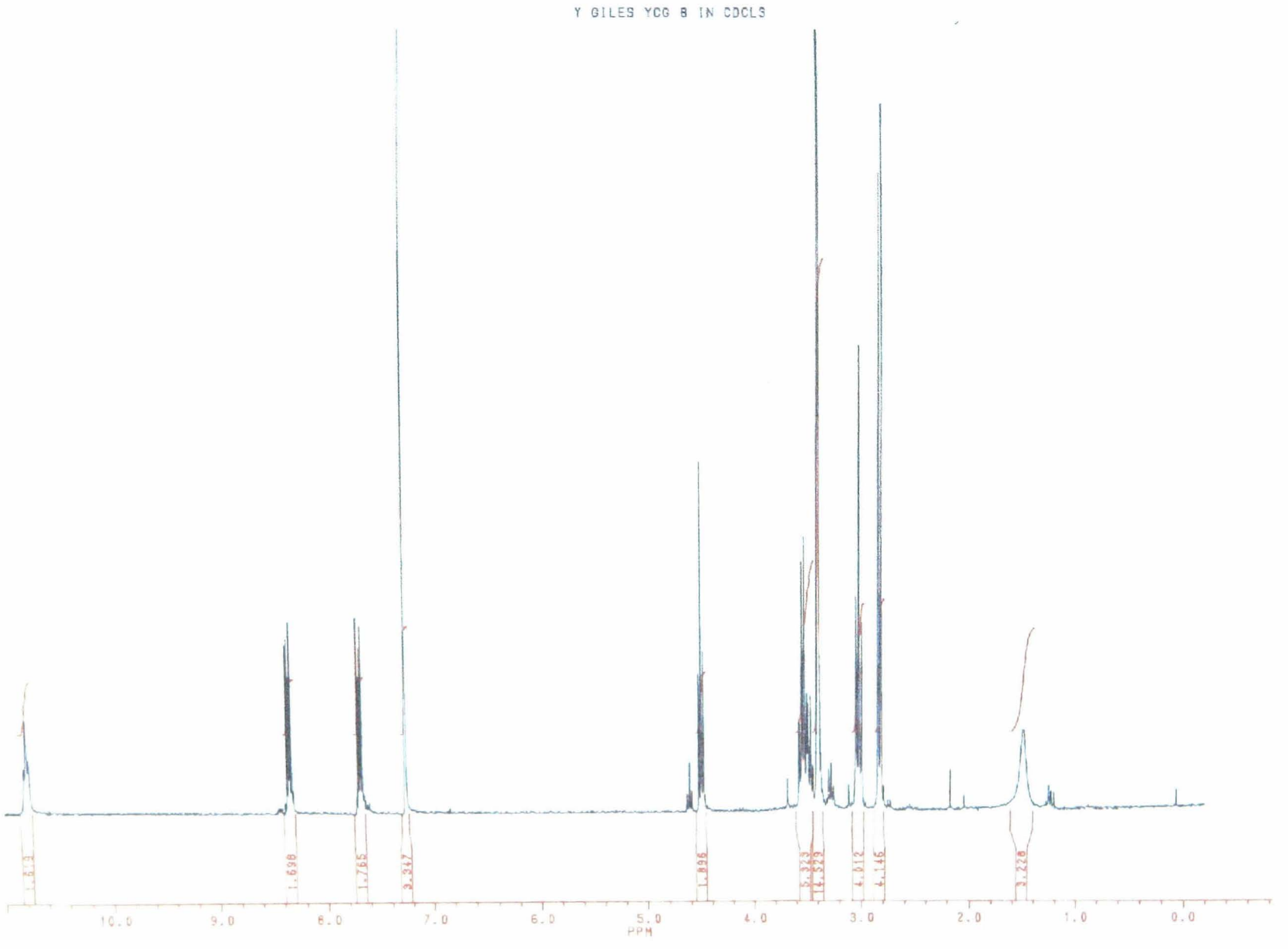


1,5-diamino-4,8-dihydroxy-anthracene-9,10-dione

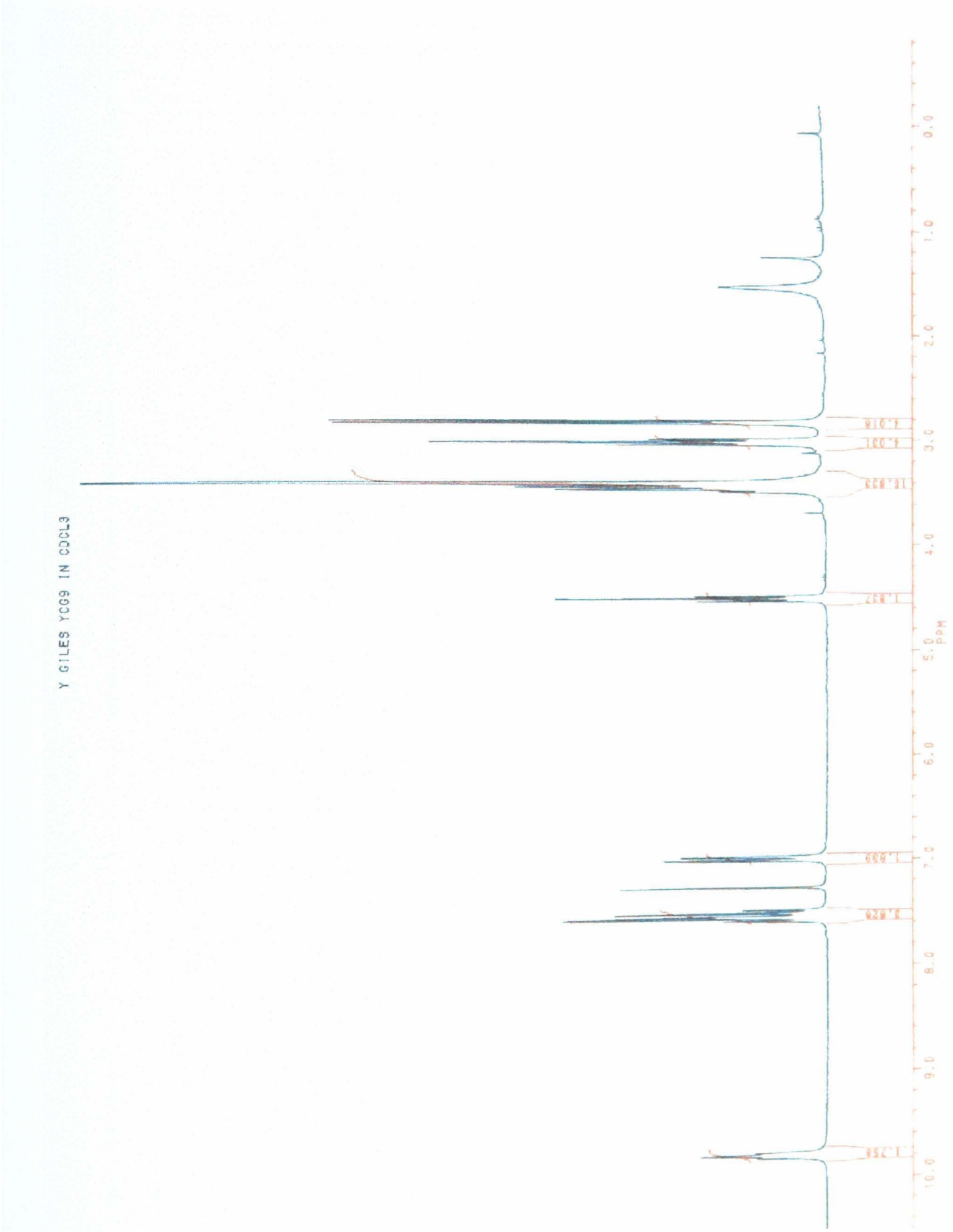
2,3-dihydro (leuco) form

A4.1 NMR FOR YCG7

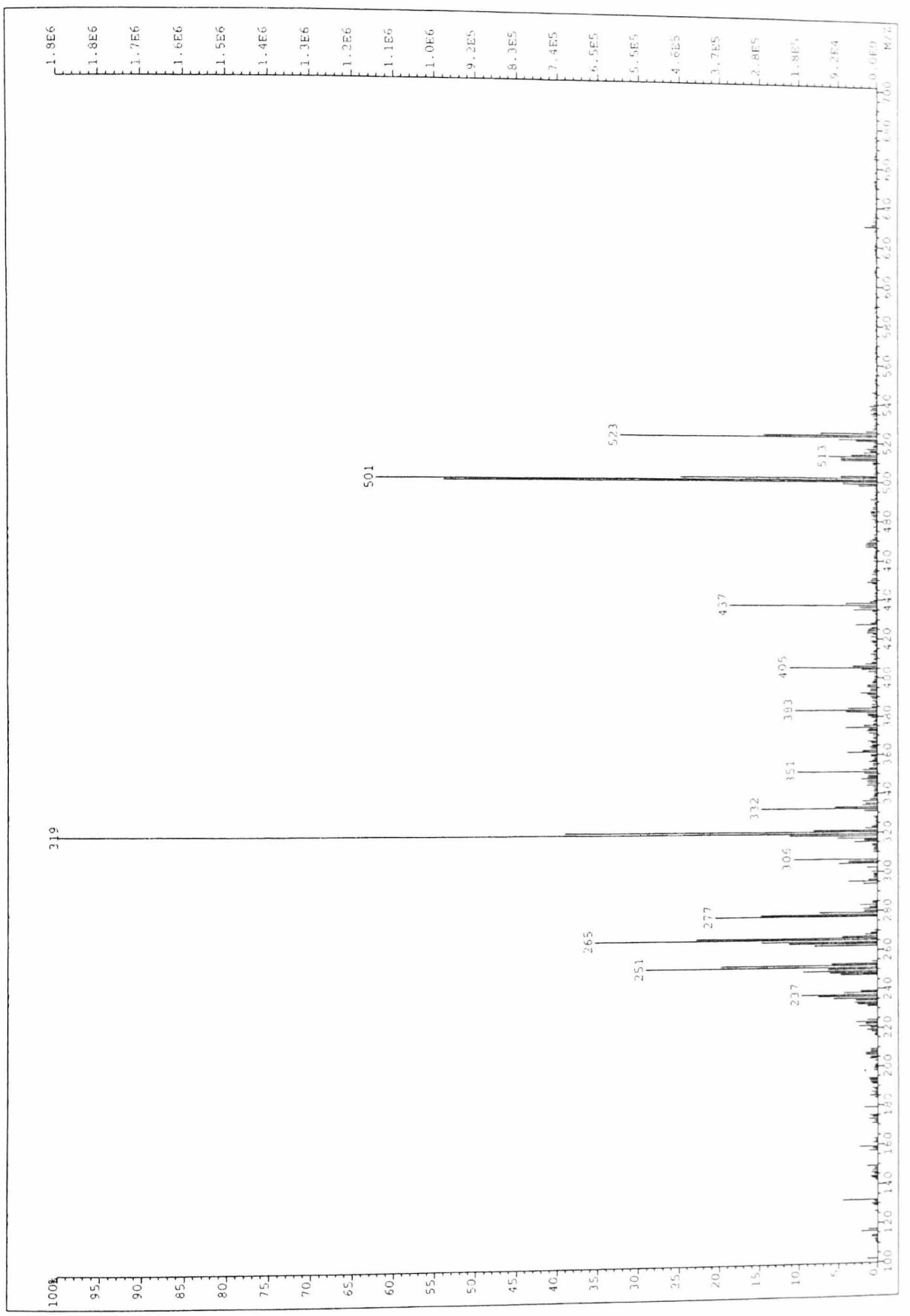



Y GILES YCG 8 IN CDCL3
1.698
1.765
3.847
1.896
3.228
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM

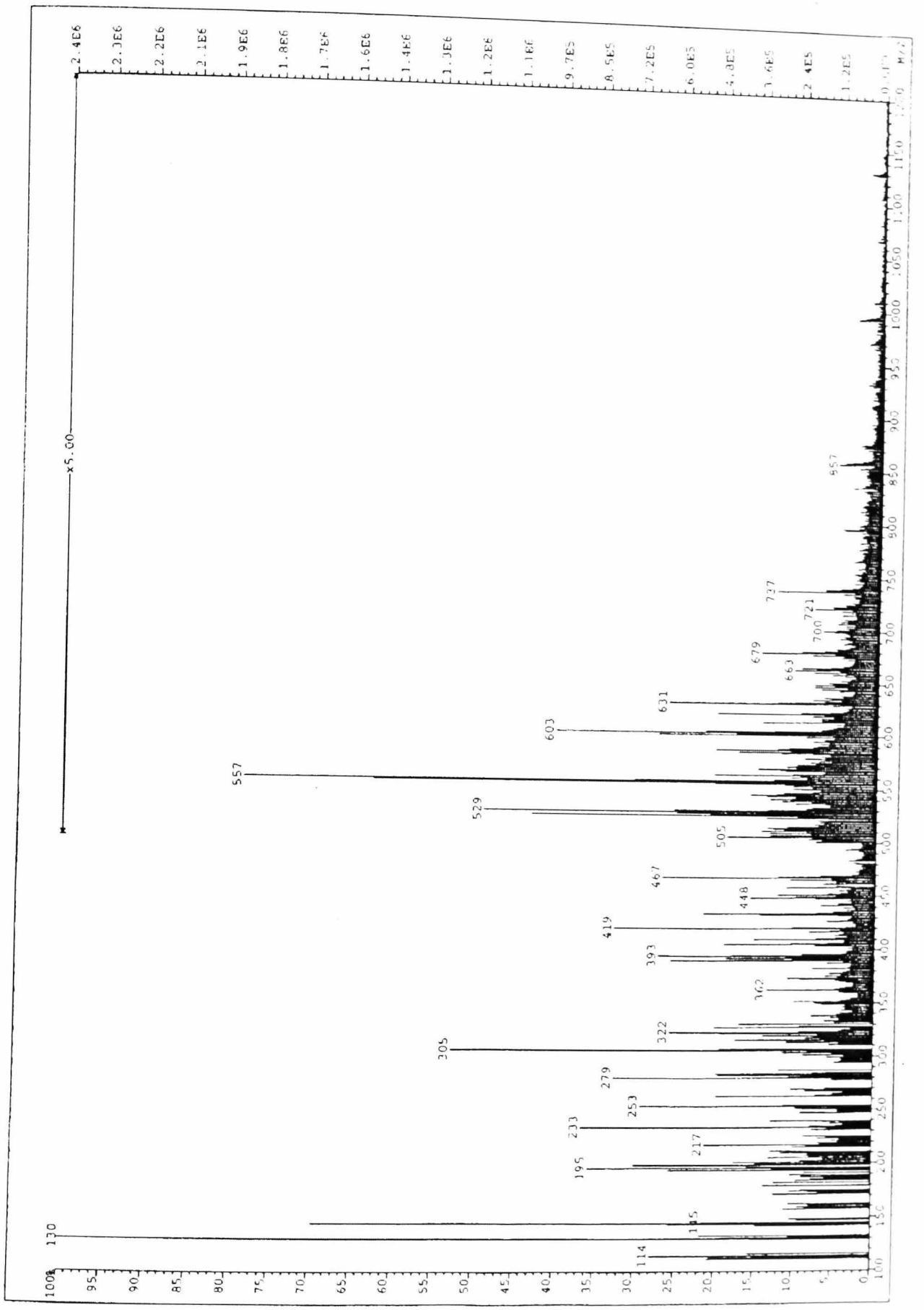
A4.3 NMR FOR YCG9



A4.4 MASS SPECTRUM FOR YCG8



A4.5 MASS SPECTRUM FOR YCG4



A5: SPECTRAL DETERMINATION OF CYTOCHROME P-450 CONTENT OF BALB C MOUSE LIVER MICROSOMES

A homogeneous suspension of Balb C mouse liver microsomes was diluted with ice-cold 0.1M Tris buffer, pH7.4, containing 20% v/v glycerol to give approximately 2mgml^{-1} protein. The suspension was divided equally between two matched glass cuvettes, and approximately 1mg of sodium dithionite was added to each, stirring their contents gently. The absorbance was then recorded between 400 and 500nm. The sample cuvette was immediately bubbled with carbon monoxide (BDH, Leicester, U.K) for 1 min (at a rate of approximately one bubble/sec) and the samples were re-scanned between 400 and 500nm.

When the haem iron of cytochrome P-450 was reduced by sodium dithionite and complexed with CO, a characteristic spectrum was obtained, with an absorbance maximum of 450nm. The concentration of cytochrome P-450 (nmolmg^{-1} protein) was calculated from a linear graph obeying Beer-Lambert's Law, using an extinction coefficient of 91nM^{-1}cm^{-1} (see Gibson & Skett, 1986). The cytochrome P-450 content was 0.46 nmolmg^{-1} protein.

A6: DETERMINATION OF REACTION KINETICS FOR THE HYDROLYSIS OF THE ACETALANTHRAQUINONES

A6.1 THE KINETIC EQUATIONS FOR ZERO-, FIRST- AND SECOND ORDER REACTIONS

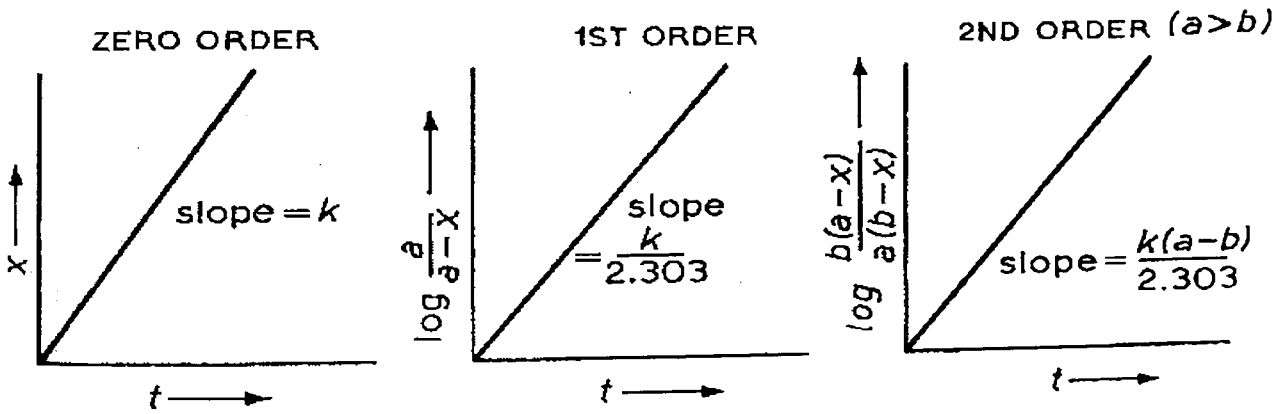
For a zero order reaction: $x = kt$

For a first order reaction: $\log [a/(a-x)] = kt/2.303$

For a second order reaction, when a>b: $\log [b(a-x)/a(b-x)] = k(a-b)t/2.303$

In these equations, x is the concentration of the rate-determining reactant utilised during time *t* of the reaction. The initial concentration of reactant A is represented by *a*, so that after time *t* the remaining concentration of *a* is represented by *(a-x)*. Similarly, when a second reactant B is rate-determining, its initial concentration is represented by *b*, and after time *t* the remaining concentration of *b* is represented by *(b-x)*.

By choosing the appropriate ordinates, the kinetic equations above can represented as straight line plots against time, which can then be used to calculate both the order and the rate constant (k) of the reaction:



In order to calculate k, the plot obtained must be linear. If a straight line plot is not obtained, then the reaction does not follow that particular order overall.

A6.2 DETERMINATION OF THE OVERALL ORDER OF THE REACTION FOR THE HYDROLYSIS OF THE ACETALANTHRAQUINONES

(a) Plots obtained using the kinetic equation for a zero order reaction

For the hydrolysis reaction of acetalanthraquinones, the plots generated using the kinetic equation for a zero order reaction were not linear, demonstrating that the reaction was not zero order overall (figures 1 to 3 show the results obtained for YCG7, YCG8 and YCG9 at 90°C).

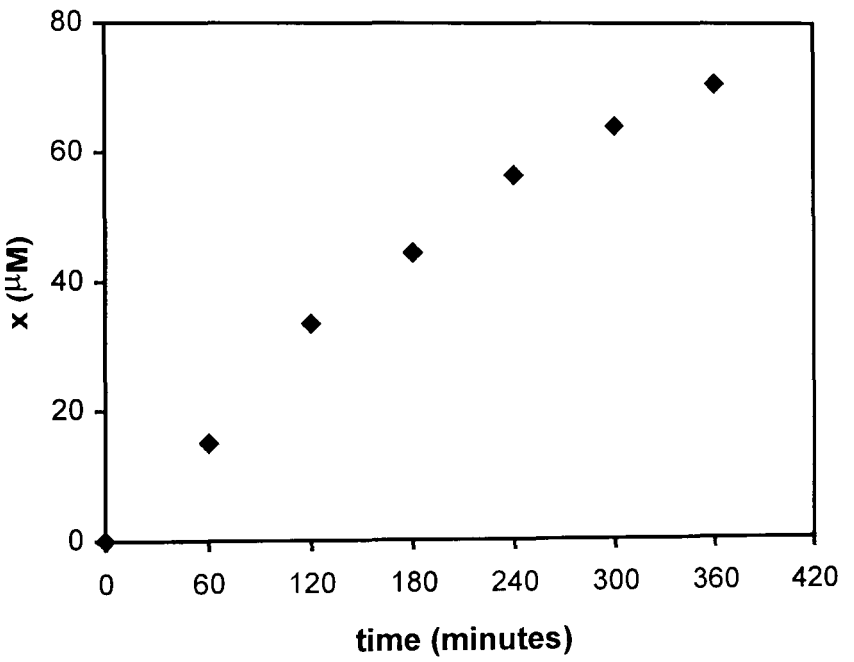


Figure 1: Plot obtained for the hydrolysis of YCG7 using the kinetic equation for a zero order reaction

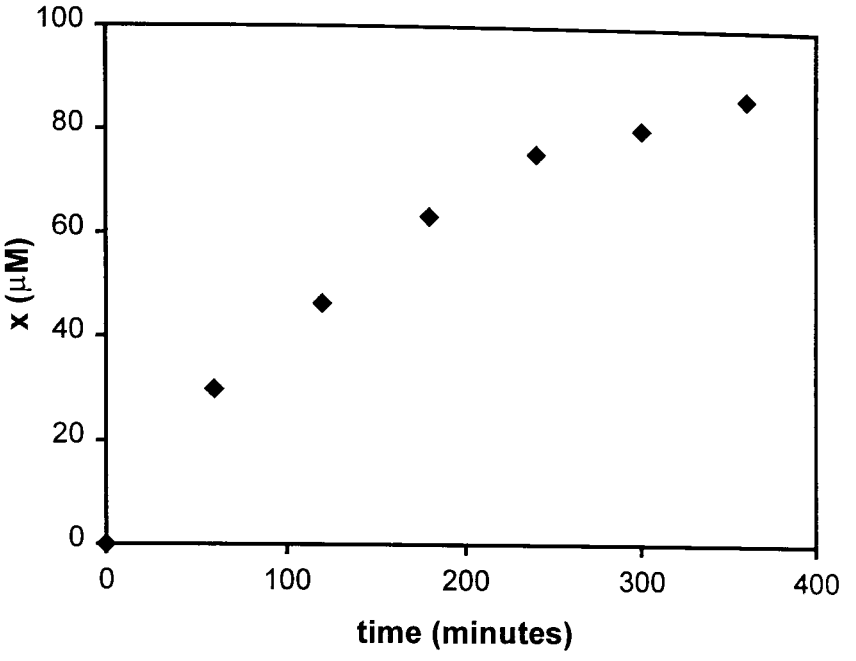


Figure 2: Plot obtained for the hydrolysis of YCG8 using the kinetic equation for a zero order reaction

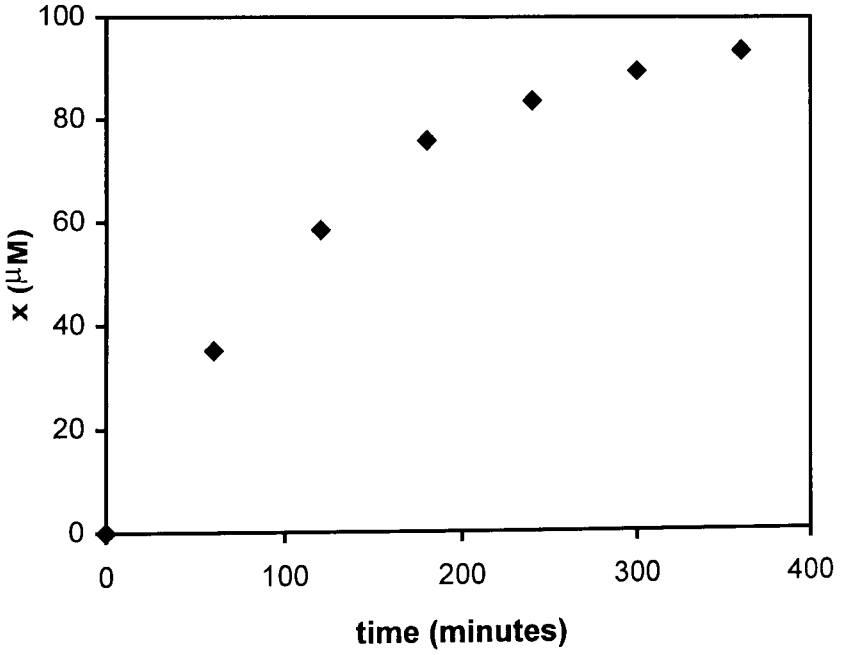


Figure 3: Plot obtained for the hydrolysis of YCG9 using the kinetic equation for a zero order reaction

(b) Plots obtained using the kinetic equation for a first order reaction

Taking *a* as the initial concentration of the drug (100μM) and *x* as the concentration of drug utilised during time *t* of the reaction, a plot of log a/a-x vs t produced a straight line, demonstrating that the hydrolysis reaction followed first order kinetics:

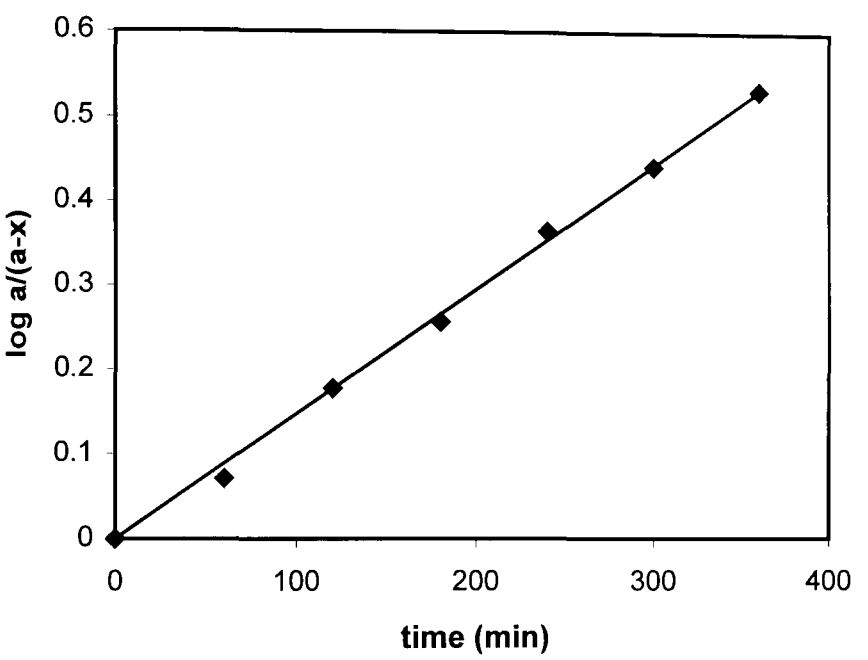


Figure 4: Plot obtained for the hydrolysis of YCG7 using the kinetic equation for a first order reaction

Because hydrolysis of the acetalanthraquinones is likely to occur by the S_N2 mechanism (see section 4.4.1), the reaction would usually be expected to follow second order kinetics overall. However, S_N2 reactions in which one reactant is in large excess usually display *pseudo* first order kinetics, because only one reactant is rate-determining. Thus, as water was in great excess throughout the hydrolysis reaction, it followed *pseudo* first order kinetics, where the rate was only dependent on the concentration of drug.

A7: MAINTENANCE OF V79 CELLS

A7.1 PREPARATION OF RPMI 1640 CULTURE MEDIUM

Powdered RPMI 1640 medium (supplied by Gibco BRL, UK) consisted of 25mM N-2- hydroxyethylpiperazine-N'-2-ethane sulfonic acid buffer (HEPES) and L-glutamine. The powder was added to 1 litre of distilled water and the pH adjusted to 7.2 using NaOH. After sterile filtration into pre-autoclaved bottles using a Millipore bottle filter, the medium was incubated for a minimum of three days at 37°C before checking for contamination. The medium was supplemented immediately prior to use.

A7.2 PROCEDURE FOR PASSAGING V79 CELLS

RPMI medium was removed from culture flasks and replaced with EDTA (5ml at 0.1% in DPBS) for 30 sec, ensuring contact with all cells. The flasks were then incubated vertically at 37°C for approximately 15 min, or until the cells detached from the flask surface. The cells were then resuspended in fully supplemented RPMI medium (5ml) and any cell clumps were dispersed by passing the cells through a 19 gauge needle 2-3 times. The cells were reseeded in culture flasks to give a five-fold reduction in original cell concentration.

A7.3 COUNTING OF V79 CELLS USING A PARTICLE COUNTER

Cells were passaged and collected in RPMI medium as described in section A7.2 and after dispersing any cell clumps, a 1 in 20 dilution of the cell suspension was made using saline. After thorough mixing, the number of cells in the saline suspension was determined using a particle counter Model ZM (Coulter Electronics Ltd, Luton. U.K). The cell suspension was adjusted with fully supplemented RPMI medium to give the required cell concentration(s). These were recounted to ensure accurate dilution.

A8: ABBREVIATIONS

AML: Acute myelocytic leukaemia

ALL: Acute lymphocytic leukaemia

ATP: Adenosine triphosphate

BSA: Bovine serum albumin

CHF: Congestive heart failure

CLL: Chronic lymphocytic leukaemia

DMSO: Dimethyl sulfoxide

DPBS: Dulbecco's phosphate buffered saline

EDTA: Ethylenediaminetetraacetic acid

E.I: Electron impact

EGTA: Ethylenebis(oxyethylenenitrilo)tetraacetic acid

FCS: Foetal calf serum

FTIR: Fourier transform infra-red

G-CSF: Granulocyte colony-stimulating factor

KDNA: Kinetoplast DNA

m-AMSA: 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide

MFO: Mixed function oxidase

NADPH: Nicotinamide adenine dinucleotide phosphate

NSCLC: Non-small cell lung cancer

PMSF: Phenyl methane sulphonyl fluoride

SCLC: Small cell lung cancer

SDS: Sodium dodecyl sulphate

TEA: Triethyl-amine

TNQ: Tetrahydronaphthoquinoxaline

Topo II: Topoisomerase II

Tris: 2-Amino-2-(hydroxymethyl)-1,3-pronanediol

BIBLIOGRAPHY

Abraham, R., Basser, R. L. and Green, M. D. (1996). A risk-benefit assessment of anthracycline antibiotics in antineoplastic therapy. Drug Safety 15(6): 406-429.

Acton, E. M., Wasserman, K. and Newman, R. A. (1988). Morpholinyl anthracyclines. In: Anthracycline and Anthracenedione-Based Anti-cancer Agents. pp 56-101. Ed. J. W. Lown. Published by Elsevier, New York.

Acton, E. M., Tong, G. L., Mosher, C. W and Wolgemuth, R. L. (1984). Intensely potent morpholinyl anthracyclines. Journal of Medicinal Chemistry 27: 638-645.

Adamson, R. H. (1974). Daunomycin and adriamycin: a hypothesis concerning anti-tumour activity and cardiotoxicity. Cancer Chemotherapy Reports 58: 293-294.

A'Hern, R. P. and Gore, M E. (1995). Impact of doxorubicin on survival in advanced ovarian cancer. Journal of Clinical Oncology 13: 726-732.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K. and Watson, J. D. (1983). Molecular Biology of the Cell. Published by Garland, Inc., New York, U.S.A.

Alderton, P. M., Gross, J. and Green, M. D. (1992). Comparative study of doxorubicin, mitoxantrone, and epirubicin in combination with ICRF-187 (ADR-529) in a chronic cardiotoxicity animal model. Cancer Research 52: 194-201.

Alley, M. C., Scudiero, D. A., Monks, A., Hursey, M. L., Czerwinski, M. J., Fine, D. L., Abboott, B. J., Mayo, J. G., Shoemaker, R. H. and Boyd, M. R. (1988). Feasibility of drug screening with panels of human tumour cell lines using a microculture tetrazolium assay. Cancer Research 48: 589-601.

Almquist, K. C., Loe, D. W., Hipfner, D. R., Mackie, J. E., Cole, S. P. C. and Deeley, R. G. (1995). Characterization of the Mr190,000 multidrug resistance protein (MRP) in drug-selected and transfected human tumor cells. Cancer Research 55: 102-110.

Andersson, B. S., Eksborg, S., Vidal, R. F., Sundberg, M. and Carlsberg, M. (1999). Anthraquinone-induced cell injury: acute toxicity of carminomycin, epirubicin, idarubicin and mitoxantrone in isolated cardiomyocytes. Toxicology 135(1): 11-20.

Avramis, V. (1982). Pharmacokinetics of dihydroxyanthracenedione (DHAD) and its metabolites in rats. Pharmacologist 24: 241.

Bachur, N. R., Yu, F., Johnson, R., Hickey, R., Wu, Y. and Malkas, L. (1992). Helicase inhibition by anthracycline anticancer agents. Molecular Pharmacology 41: 993-998.

Baggetto, L. (1997). Biochemical, genetic, and metabolic adaptations of tumour cells that express the typical multidrug-resistance phenotype. Reversion by new therapies. Journal of Bioenergetics and Biomembranes 29:(4): 401-413.

Baguley, B. C., Holdaway, K. M. and Fray, L. M. (1990). Design of DNA intercalators to overcome topoisomerase II-mediated multidrug resistance. Journal of the National Cancer Institute 82: 398-402.

Bailly, J. D., Skladanowski, A., Bettaieb, A., Mansat, V., Larsen, A. K. and Laurent, G. (1997). Natural resistance of acute myeloid leukemia cell lines to mitoxantrone is associated with lack of apoptosis. Leukemia 11(9): 1523-1532.

Bailly, C., Routier, S., Bernier, J.-L. and Waring, M. J. (1996). DNA recognition by two mitoxantrone analogues: influence of the hydroxyl groups. FEBS Letters 379: 269-272.

Bakker, M., Droz, J. P., Hanauske, A. R., Verweij, J., van Oosterom, A. T., Groen, H. J. M., Pacciarini, M. A., Domenigoni, L., van Weissenbruch, F., Paniezzola, E. and de Vries, E. G. E. (1998). Broad phase II and pharmacokinetic study of methoxy-morpholino doxorubicin (FCE 23762-MMRDX) in non-small-cell lung cancer, renal cancer and other solid tumour patients. British Journal of Cancer 77(1): 139-146.

Banerjee, D., Zhao, S. C., Li, M. X., Schweitzer, B. I., Mineishi, S. and Bertino, J. R. (1994). Gene-therapy utilizing drug-resistance genes- a review. Stem Cells 12(4): 378-385.

Barrett, J. F., Sutcliffe, J. A. and Gootz, T. D. (1990). *In vitro* assays used to measure the activities of topoisomerases. Antimicrobial Agents and Chemotherapy 34(1): 1-7.

Basra, J., Wolf, C. R., Brown, J. R and Patterson, L. H (1985). Evidence for human liver microsome free radical formation by doxorubicin and mitoxantrone. Anti-cancer Drug Design 1: 45-52.

Bassan, R., Lerede, T. and Rambaldi, A. (1996). The role of anthracyclines in adult acute lymphoblastic leukaemia. Leukemia 10(S2): S58-S61.

Bauman, P. A., Dalton, W. S., Anderson, J. M. and Cress, A. E. (1994). Expression of cytokeratin confers multiple-drug resistance. Proceedings of the National Academy of Sciences of the U.S.A. 91(12): 5311-5314.

Beck, W. T., Danks, M. K., Wolverton, J. S., Kim, R. and Chen, M. (1993). Drug resistance associated with altered topoisomerase II. Advances in Enzyme Regulation 33: 113-127.

Beck, W. T (1990). Srategies to circumvent multidrug resistance due to P-glycoprotein or to altered DNA topoisomerase II. Bulletin du Cancer 77: 1131-1141.

Beck, W. T., Cirtain, M. C., Danks, M. K., Felsted, R. L. Safa, A. R., Wolverton, J. S., Suttle, D. P. and Trent, J. M. (1987). Pharmacological, molecular and cytogenetic analysis of "atypical" multidrug-resistant human leukemic cells. Cancer Research 47: 5455-5460.

Bedford, J. S. and Mitchell, J. B. (1974). The effect of hypoxia on the growth and radiation response of mammalian cells in culture. British Journal of Radiology 47: 687-696.

Beedham, C. (1997). The role of non-P450 enzymes in drug oxidation. Pharmacy World and Science 19(6): 255-263.

Begleiter A. and Johnston, J. B. (1985). DNA cross-linking activity of 3'-(3-cyano-4-morpholinyl)-3'-deaminoadriamycin in HT-29 human colon carcinoma cells *in vitro*. Biochemical and Biophysical Research Communications 131: 336-338.

Bell, A., Brown, J R. and Neidle, S. (1989). Thermodynamic studies on the interactions of di-substituted anthraquinones with DNA. Biochemical pharmacology 38(1): 216-217.

Bellamy, W. T., Dalton, W. S., Kailey, J. M., Gleason, M. C., McCloskey, T. M., Dorr, R. T. and Alberts, D. S. (1988). Verapamil reversal of doxorubicin resistance in multodrug-resistant human myeloma cells and association with drug accumulation and DNA damage. Cancer Research 48: 6365-6370.

Bellosillo, B., Colomer, D., Pons, G. and Gil, J. (1998). Mitoxantrone, a topoisomerase II inhibitor, induces apoptosis of B-chronic lymphocytic leukaemia cells. British Journal of

Haematology 100: 142-146.

Berger, J. M., Gamblin, S. J., Harrison, S. C. and Wang, J. C. (1996). Structure and mechanism of DNA topoisomerase II. Nature 379: 225-232.

Bhalla, K., Ibrado, A. M., Tourkina, E., Tang, C., Grant, S., Bullock, G., Huang, Y., Ponnathpur, V. and Mahoney, M. E. (1993). High-dose mitoxantrone induces programmed cell death or apoptosis in human myeloid leukemia cells. Blood 82: 3133-3140.

Bierlack, S. S., Erttmann, R., Winkler, K. and Landbeck, G. (1989). Doxorubicin: effect of different schedules on toxicity and anti-tumor efficacy. European Journal of Cancer and Clinical Oncology 25: 873-882.

Binaschi, M., Capranico, G., De Isabella, P., Mariani, M., Supino, R., Tinelli, S. and Zunino, F. (1990). Comparison of DNA cleavage induced by etoposide and doxorubicin in two human small-cell lung cancer cell lines with different sensitivities to topoisomerase II inhibitors. International Journal of Cancer 45: 347-352.

Blake, A. and Peacocke, A. R. (1968). The interaction of aminoacridines with nucleic acids. Biopolymers 6: 1225-1253.

Blanz, J., Mewes, K., Ehninger, G., Proksch, B., Waidelich, D., Greger, B. and Zeller, K-P. (1991a). Evidence for oxidative activation of mitoxantrone in human, pig, and rat. Drug Metabolism and Disposition 19(5): 871-880.

Blanz, J., Mewes, K., Ehninger, G., Proksch, B., Greger, B., Waidelich, D. and Zeller, K-P. (1991b). Isolation and structure elucidation of urinary metabolites of mitoxantrone. Cancer Research 51: 3427-3433.

Bloom, S. M. and Hutton, R. F. (1963). Nuclear magnetic resonance studies of tautomerism: loss of aromaticity in "leuco"-hydroxy and aminoanthraquinones. Tetrahedron Letters: 1993-1997.

Bodley, A., Liu, L. F., Israel, M., Seshadri, R., Koseki, Y., Giuliani, F. C., Kirschenbaum, S., Silber, R. and Potmesil, M. (1989). DNA topoisomerase II-mediated interaction of doxorubicin and daunorubicin congeners with DNA. Cancer Research 49: 5969-5978.

Boege, F., Kjeldsen, E., Gieseler, F., Alsner, J. and Biersack, H. (1993). A drug- resistant variant of topoisomerase IIα in human HL-60 cells exhibits alterations in catalytic pH optimum, DNA binding and sub-nuclear distribution. European Journal of Biochemistry 218: 575-584.

Boesen, J. J. B., Nooter, K. and Valerio, D. (1993). Circumvention of chemotherapy-induced myelosuppression by transfer of the MDR1 gene. Biotherapy 6(4): 291-302.

Bohr, V. A., Phillips, D. H. and Hanawalt, P. C. (1987). Heterogeneous DNA damage and repair in the mammalian genome. Cancer Research 47: 6426-6436.

Boiron, J.-M., Belloc, F., Montastruc, M., Conymakhoul, P., Dumain, P., Marit, G., Mahon, F. X., Puntous, M., Lopez, F., Lacombe, F. and Reiffers, J. (1994). Flow cytometric study of idarubicin and daunorubicin accumulation and the effect of verapamil in leukaemic cell lines and fresh cells from patients with acute non-lymphoblastic leukaemia. Leukaemia Research 18: 313-318.

Bonnadonna, G., Gianni, L., Santoro, A., Bonfante, V., Bidoli, P., Casali, P., Demicheli, R. amd Valagussa, P. (1993). Drugs ten years later: epirubicin. Annals of Oncology 4: 359-369.

Borst, P., Schinkel, A. H., Smit, J. J. M., Wagenaar, E., van Deemter, L., Smith, A. J., Eijdems, E. W. H. M., Baas, F. and Zaman, G. J. R. (1993). Classical and novel forms of multidrug resistance and the physiological functions of P-glycoproteins in mammals. Pharmacology and Therapeutics 60: 289-299.

Bosron, W. F and Li T-K. (1980). Alcohol dehydrogenase. In: Enzymatic basis of detoxification 1: 231-248. Ed. W. B. Jakoby. Published by Academic Press.

Boucek, , R. J. Jr, Olsen, R. D., Brenner, D. E., Oganuburnmi, E.M., Inui, M. and Fleischer, S. (1987). The major metabolite of doxorubicin is a potent inhibitor of membrane-associated ion pumps. A correlative study of cardiac muscle with isolated membrane fractions. Journal of Biological Chemistry 262: 15851-15856.

Bowers, C., Adkins, D., Dunphy, F., Harrison, B., Lemaistre, C. F and Spitzer, G. (1993). Dose escalation of mitoxantrone given with thiotepa and autologous bone marrow transplantation for metastatic breast cancer. Bone Marrow Transplant 12: 525-530.

Bristow, M. R., Thompson, P. D., Martin, R. P., Mason, J. W., Billingham, M. E. and Harrison, D. C. (1978). Early anthracycline cardiotoxicity. American Journal of Medicine 65: 823-832.

Brown, J. M. and Siim, B. G. (1996). Hypoxia-specific cytotoxins in cancer therapy. Seminars in Radiation Oncology 6: 22-36.

Brown, G. A., McPherson, J. P., Gu, L., Hedley, D. W., Toso, R., Deuchars, K. L., Freedman, M. H. and Goldenberg, G. J. (1995). Relationship of DNA topoisomerase IIα and β expression to cytotoxicity of antineoplastic agents in human acute lymphoblastic leukemia cell lines. Cancer Research 55: 78-82.

Brown, J. M. (1993). SR 4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumours. British Journal of Cancer 67: 1163-1170.

Brown, J. M. (1979). Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism for reoxygenation. British Journal of Radiology 52: 650-656.

Capranico, G., Binaschi, M., Borgnetto, E. M., Zunino, F. and Palumbo, M. (1997). A protein-mediated mechanism for the DNA sequence-specific action of topoisomerase II poisons. Trends in Pharmacological Sciences 18: 323-329.

Capranico, G., Supino, R., Binaschi, M., Capolongo, L., Grandi, M., Suarato, A. and Zunino, F. (1994). Influence of structural modifications at the 3' and 4' positions of doxorubicin on the drug ability to trap topoisomerase II and to overcome drug resistance. Molecular Pharmacology 45: 908-915.

Capranico, G., De Isabella, P., Tinelli, S., Bigioni, M. and Zunino, F. (1993). Similar sequence specificity of mitoxantrone and VM-26 stimulation of *in vitro* DNA cleavage by mammalian DNA topoisomerase II. Biochemistry 32: 3038-3046.

Capranico, G., Kohn, K. W. and Pommier, Y. (1990). Local sequence requirements for DNA cleavage by mammalian topoisomerase II in the presence of doxorubicin. Nucleic Acids Research 18: 6611-6619.

Capranico, G., De Isabella, P., Penco, S., Tinelli, S. and Zunino, F. (1989). Role of DNA breakage in cytotoxicity of doxorubicin, 9-deoxydoxorubicin and 4-demethyl-6-

deoxydoxorubicin in murine leukaemia P388 cells. Cancer Research 49: 2022-2027.

Carmichael, J., DeGraff, W. G., Gazdar, A. F., Minna, J. D. and Mitchell, J. B. (1987). Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Research 47: 936-942.

Caron, P. R. and Wang, J. C. (1993). DNA topoisomerases as targets of therapeutics: a structural overview; in Molecular Biology of DNA topoisomerases: 1-18. Ed Andoh, T., Ikeda, H. and Oguro, M. CRC press, Boca Raton, Florida, U. S. A.

Cassinelli, G., Configliacchi, E., Penco, S., Rivola, G., Arcamone, F., Pacciarini, A. and Ferrari, L. (1984). Separation, charaterization and analysis of epirubicin (4'-epidoxorubicin) and its metabolites from human urine. Drug Metabolism and Disposition 12: 506-510.

Catapano, C. V., Carbone, G. M. R., Pisani, F., Qiu, J. and Fernandes, D. J. (1997). Arrest of replication fork progression at sites of topoisomerase II-mediated DNA cleavage in human leukaemia CEM cells incubated with VM-26. Biochemistry 36: 5739-5748.

Cersosimo, R. J. (1992). Idarubicin: an anthracycline antineoplastic agent. Clinical Pharmacology 11: 152-167.

Chaires, J. B., Dattagupta, N. and Crothers, D. M. (1982). Studies on interaction of anthracycline antibiotics and deoxyribonucleic acid: equilibrium binding studies on interaction of daunomycin with deoxyribonucleic acid. Biochemistry 21: 3933-3940.

Chaney, S. G and Sancar, A. (1996). DNA repair enzymatic mechanisms and relevence to drug response. Journal of the National Cancer Institute 8(19): 1346-1360.

Chang, C. W., Barber, L., Ouyang, C. Masin, D., Bally, M. B. and Madden, T. D. (1997). Plasma clearance, biodistribution and therapeutic properties of mitoxantrone encapsulated in conventional and sterically stabilized liposomes after intravenous administration in BDF1 mice. British Journal of Cancer 75: 169-177.

Chegini, N. and Safa, A. R. (1987). Influence of mitoxantrone on nucleolar function in MDA-MB-231 human breast cancer cell line. Cancer Letters 37: 327-336.

Chen, K.-X., Gresh, N. and Pullman, B. (1986). A theoretical investigation on the sequence-

selective binding of mitoxantrone to double stranded DNA. Nucleic Acids Research 14: 3799-3812.

Chen, G. L., Yang, L., Rowe, T. C., Halligan, B. D., Tewey, K. M. and Liu, L. F. (1984). Non-intercalative antitumour drugs interfer with the breakage-reunion reaction of mammalian DNA topoisomerase II. Journal of Biological Chemistry 259: 13560-13566.

Cheng, C. C. and Zee-Cheng, R. K.-Y. (1983). The design, synthesis and development of a new class of potent antineoplastic anthraquinones. Progress in Medicinal Chemistry 20: 83-118.

Chiccarelli, F. S., Morrison, J. A., Cosulich, D, B., Perkinson, N. A., Ridge, D. N., Sum, F. W., Murdock, K. C., Woodward, D. L. and Arnold, E. T. (1986). Identification of human urinary mitoxantrone metabolites. Cancer Research 46: 4858-4861.

Chow, K-C. and Ross, W. E. (1987). Topoisomerase-specific drug sensitivity in relation to cell cycle progression. Molecular and Cellular Biology 7(9): 3119-3123.

Cholerton, S., Daly, A. K and Idle, J. R. (1992). The role of individual cytochromes P-450 in drug metabolism and clinical response. Trends in Pharmacological Sciences 13: 434-439.

Ciarrocchi, G., Montecucco, A., Pedrali-Noy, G. and Spadari, S. (1988). A novel target in DNA metabolism for cytotoxic drugs 37(9): 1803-1804.

Coleman, C. N. (1988). Hypoxia in tumours: A paradigm for the approach to biochemical and physiologic heterogeneity. Journal of the National Cancer Institute 80: 310-317.

Coley, H, M., Amos, W. B., Twentyman, P. R and Workman, P. (1993). Examination by laser scanning confocal fluorescence imaging microscopy of the subcellular localization of anthracyclines in parent and multidrug resistant cell lines. British Journal of Cancer 67: 1316-1323.

Cornarotti, M., Tinelli, S., Willmore, E., Zunino, F., Fisher, L. M., Austin, C. A. and Capranico, G. (1996). Drug sensitivity and sequence specificity of human recombinant DNA topoisomerases II alpha (p170) and beta (p180). Molecular Pharmacology 50(6): 1463-1471.

Cortes, F., Pinero, J. (1994). Synergistic effect of inhibitors of topoisomerase I and topoisomerase II on chromosome damage and cell killing in cultured Chinese hamster ovary

cells. Cancer Chemotherapy and Pharmacology 34(5): 411-415.

Coukell, A. J. and Faulds, D. (1997). Epirubicin: an updated review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the management of breast cancer. Drugs 53(3): 453-482.

Cox, P. J., Farmer, P. B. and Jarman, M. (1976a). Symposium on the metabolism and mechanism of action of cyclophosphamide. Cancer Treatment Reports 60: 299-525.

Cox, P. J., Phillips, B. J. and Thomas, P. (1976b). Studies on the selective action of cyclophosphamide: inactivation of the hydroxylated metabolite by tissue-soluble enzymes. Cancer Treatment Reports 60: 321-326.

Cress, A. E., Roberts, R. A., Bowden, G. T. and Dalton, W. S. (1988). Modification of keratin by the chemotherapeutic drug mitoxantrone. Biochemical Pharmacology 37: 3043-3046.

Crothers, D. M. (1968). Calculation of binding isotherms for heterogeneous polymers. Biopolymers 6: 575-584.

Crown, J., Wasserheit, C., Hakes, T., Fennelly, D., Reich, L., Moore, M. and Schneider, J. (1992). Rapid delivery of multiple high-dose chemotherapy courses with granulocyte colony-stimulating factor and peripheral blood derived hematopoietic progenitor cells. Journal of the National Cancer Institute 84: 1935-1936.

Curtis, J. E., Minden, M. D., Minkin, S. and McCulloch, E. A. (1995). Sensitivities of AML blast stem cells to idarubicin and daunorubicin: a comparison with normal hematopoietic progenitors. Leukemia 9: 396-404.

Damle, B. and Desai, P. (1994). Dipyridamole reverses the resistance to topoisomerase II inhibitors but not antimicrotubule agents in multidrug-resistant melanoma cells. Oncology Research 6(2): 49-57.

Danesi, R., Agen, C., Grandi, M., Nardini, V., Bevilacqua, G. Del Tacca, M. (1993). 3'-deamino-3'-(2-methoxy-4-morpholinyl)-doxorubicin (FCE 23762): A new anthracycline derivative with enhanced cytotoxicity and reduced cardiotoxicity. European Journal of Cancer 29A(11): 1560-1565.

Dang, C. V., Lewis, B. C., Dolde, C., Dang, G. and Shim, H. (1997). Oncogenes in tumor metabolism, tumorigenesis, and apoptosis. Journal of Bioenergetics and Biomembranes 29:(4): 345-354.

Danks, M. K., Qiu, J., Catapano, C. V., Schmidt, C. A., Beck, W. T. and Fernandes, D. J. (1994). Subcellular distribution of the α and β topoisomerase II-DNA complexes stabilized by VM-26. Biochemical Pharmacology 48: 1785-1795.

Danks, M. K., Beck, W. T. and Suttle, D. P. (1993). Topoisomerase IIα mutation in leukemic cells from a patient with lineage switch AML. Proceedings of the American Association for Cancer Research 34: abstract 1982.

D'Arpa, P., Schneider, E. and Liu, L. F. (1992). Mechanism of tumour cell killing by topoisomerase poisons, in Drug Resistance as a Biochemical Target in Cancer Chemotherapy: 147-163. Academic Press Inc.

D'Arpa, P., Beardmore, C. and Liu, L. F. (1990). Involvement of nucleic acid synthesis in cell killing mechanisms of topoisomerase poisons. Cancer Research 50: 6919-6924.

D'Arpa, P. and Liu, L. F. (1989). Topoisomerase-targeting antitumour drugs. Biochimica et Biophysica Acta 989: 163-177.

Davies, S. M., Robson, C. N., Davies, S. L. and Hickson, I. D. (1988). Nuclear topoisomerase II levels correlate with the sensitivity of mammalian cells to intercalating agents and epipodophyllotoxins. Journal of Biological Chemistry 263(33): 17724-17729.

De Graaf, H., Dolsma, V. W., Willemse, P. H. B., vanderGraaf, W. T. A., Sleijfer, D. T., de Vries, E. G. E. and Mulder, N. H. (1997). Cardiotoxicity from intensive chemotherapy combined with radiotherapy in breast cancer. British Journal of cancer 76(7): 943-945.

De Isabella, P., Capranico, G., Palumbo, M., Sissi, C., Krapcho, A. P. and Zunino, F. (1993). Sequence selectivity of topoisomerase II cleavage stimulated by mitoxantrone derivatives: relationships to drug DNA binding and cellular effects. Molecular Pharmacology 43: 715-721.

Deffie, A. M., Batra, J. K. and Goldenberg, G. J (1989). Direct correlation between topoisomerase II activity and cytotoxicity in adriamycin-sensitive and -resistant P388 leukaemia cell lines. Cancer Research 49: 58-62.

Denny, W. A. and Wilson, W. R. (1998). The design of selectively-activated anti-cancer prodrugs for use in antibody-directed and gene-directed enzyme prodrug therapies. Journal of Pharmacy and Pharmacology 50:387-394.

Denny, W. A. (1996). The design of selectively-activated prodrugs for cancer chemotherapy. Current Pharmaceutical Design 2: 281-294.

Denny, W. A. and Wakelin, L. P. G. (1990). Kinetics of the binding of mitoxantrone, ametantrone and analogues to DNA: relationship with binding mode and anti-tumour activity. Anti-cancer Drug Design 5: 189-200.

Dillehay, L. E., Denstman, S. C. and Williams, J. R. (1987). Cell cycle dependence of sister chromatid exchange induction by DNA topoisomerase II inhibitors. Cancer Research 47: 206-210.

Dixon, S. C., Soriano, B. J., Lush, R. M., Borner, M. M. and Figg, W. D. (1997). Apoptosis: Its role in the development of malignancies and its potential as a novel therapeutic target. Annals of Pharmacology 31(1): 76-82.

Doehmer, J. and Schmalix, W. (1994). Cytochromes P-450 expressed in V79 Chinese hamster cells. FASEB Journal 8(7): A1255.

Doroshow, J. H. (1983). Effect of anthracycline antibiotics on oxygen radical formation in rat heart. Cancer Research 43: 460-472.

Doroshow, J. H., Locher, G. Y. and Myers, C. E. (1980). Enzymatic defences of the mouse heart against reactive oxygen metabolites: alterations produced by doxorubicin. Journal of Clinical Investigation 65: 128-135.

Dorr, R. T. (1996). Cytoprotective agents for anthracyclines. Seminars in Oncology 23 (suppl. 8): 23-34.

Double, J. C. and Brown, J. R. (1975). Interaction of aminoalkylamino-anthraquinones with deoxyribonucleic acid. Journal of Pharmacy and Pharmacology 27: 502-507.

Downes, C. S., Mullinger, A. M., and Johnson, R. T. (1991). Inhibitors of topoisomerase II prevent chromatid separation in mammalian cells but do not prevent exit from mitosis.

Proceedings of the National Academy of Sciences of the U.S.A. 88: 2616-2620.

Drake, F. H., Hofman, G.A., Bartus, H. F., Mattern, M. R., Crooke, S. T. and Mirabelli, C. K. (1989). Biochemical and pharmacological properties of the p170 and p180 forms of topoisomerase II. Biochemistry 28: 8154-8160.

Drake, F. H., Zimmerman, J. P., McCabe, F. L., Bartus, H. F., Per, S. R., Sullivan, D. M., Ross, W. E., Mattern, M. R., Johnson, R. K., Crooke, S. T. and Mirabelli, C. K. (1987). Purification of topoisomerase II from amsacrine- resistant P388 leukaemia cells. Evidence for two forms of the enzyme. Journal of Biological Chemistry 262: 16739-16747.

Duran, G. E., Lau, D. H. M., Lewis, A. D. Kühl, J-S., Bämmler, T. K. and Sikic, B. I. (1996). Differential single- versus double-strand DNA breakage produced by doxorubicin and its morpholinyl analogues. Cancer Chemotherapy and Pharmacology 38: 210-216.

Duthie, S. J. and Grant, M. H. (1989). The role of reductive and oxidative metabolism in the toxicity of mitoxantrone, adriamycin and menadione in human liver derived Hep G2 hepatoma cells. British Journal of Cancer 60: 566-571.

Ehninger, G., Schuler, E., Proksch, B., Zeller, K-P. and Blanz, J. (1990). Pharmacokinetics and metabolism of mitoxantrone. Clinical Pharmacokinetics 18(5): 365-380.

Ehninger, G., Proksch, B., Heinzel, G. and Woodward, D. L. (1986). Clinical pharmacology of mitoxantrone. Cancer Treatment Reports 70(12): 1373-1378.

El Mouelhi, M., Didolkar, M., Elias, E., Guengerich, F. and Kaufmann, F. (1987). Hepatic drug-metabolizing enzymes in primary and secondary tumors of human liver. Cancer Research 47: 460-466.

Elaison, R. and Kreevoy, M. M. (1978). Kinetic hydrogen isotope effects in the concerted mechanism for the hydrolysis of acetals, ketals, and ortho esters. Journal of the American Chemical Society 100: 7037-7041.

Ellis, P. A., Smith, I. E. and Dowsett, M. (1996). Apoptosis- its role in tumour-growth and therapy. Cytopathology 7(3): 201-203.

Endicott , J. A. and Ling, V. (1989). The biochemistry of P-glycoprotein-mediated multidrug

resistance. Annual Review of Biochemistry 58: 137-171.

Enomoto, T., Saijo, M., Kimura, K. and Ui, M. (1993). Cell cycle-dependent phosphorylation of topoisomerase II and modification of its activity by phosphorylation; in Molecular Biology of DNA topoisomerases: 66-75. Ed Andoh, T., Ikeda, H. & Oguro, M. CRC press, Boca Raton.

Epstein, R. and Smith, P. J. (1988). Oestrogen-induced potentiation of DNA damage and cytotoxicity in human breast cancer cells treated with topoisomerase II-interactive antitumour drugs. Cancer Research 48: 297-303.

Estey, E., Adlakha, R., Hittelman, W. N. and Zwelling, L. A. (1987). Cell cycle stage dependent variations in drug-induced topoisomerase II mediated DNA cleavage and cytotoxicity. Biochemistry 26: 4338-4344.

Evenson, D. P., Darzynkiewicz, Z., Staiano-Coico, L., Treganos, F. and Melamed, M. R. (1979). Effects of 9,10-anthracenedione, 1,4-bis[(2-[(2-hydroxyethyl)amino]-ethyl)amino]-diacetate on cell survival and cell cycle progression in cultured mammalian cells. Cancer Research 39: 2574-2581.

Ewig, R. A. and Kohn, K. W. (1977). DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. Cancer Research 37: 2114-2122.

Facchetti, I., Grandi, M., Cucchi, P., Geroni, C., Penco, S. and Vigevani, A. (1991). Influence of lipophilicity on cytotoxicity of anthracyclines in LoVo and LoVo/Dx human cell lines. Anticancer Drug Design 6: 385-397.

Faulds, D., Balfour, J. A., Chrisp, P., and Langtry, H. D. (1991). Mitoxantrone. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in the chemotherapy of cancer. Drugs 41(3): 400-449.

Feigon, J. Denny, W. A, Leupin, W. and Kearns, D. R. (1984). Interactions of antitumour drugs with natural DNA: ^{1}H NMR studies of binding mode and kinetics. Journal of Medicinal Chemistry 27: 450-465.

Feldoff, P. W., Mirski, S. E. L., Cole, S. P. C. and Sullivan, D. M. (1994). Altered subcellular distribution of topoisomerase IIα in a drug-resistant human small cell lung cancer cell line.

Cancer Research 54: 756-762.

Feofanov, A., Sharonov, S., Fleury, F., Kudelina, I. and Nabiev, I. (1997). Quantitative confocal spectral imaging analysis of mitoxantrone within living K562 cells: intracellular accumulation and distribution of monomers, aggregates, naphthoquinoxaline metabolite, and drug-target complexes. Biophysical Journal 73: 3328-3336.

Ferrans, V. J. (1978). Overview of cardiac pathology in relation to anthracycline cardiotoxicity. Cancer Treatment Reports 62: 955-961.

Fessenden, R. J. and Fessenden, J. S. (1986). Organic Chemistry (3rd edition). Ed. S. Ewing. Published by Brookes/Cole, California, U.S.A.

Fife, T. H. (1972). General acid catalysis of acetal, ketal and ortho ester hydrolysis. Accounts of Chemical Research 5: 264-272.

Fife, T. H. and Jao, L. K. (1965). Substituent effects in acetal hydrolysis. Journal of Organic Chemistry 30: 1492-1495.

Fisher, G. R. and Patterson, L. H. (1991). DNA strand breakage by peroxidase-activated mitoxantrone. Journal of Pharmacy and Pharmacology 43: 65-68.

Fisher, B., Redmond, C., Wickerham, D.L., Bowman, D., Schipper, H., Wolmark, N., Sass, R., Fisher, E. R., Jochimsen, P., Legault-Poisson, S., Dimitrou, N., Wolter, J., Bornstein, R., Elias, E. G., Licalzi, N., Paterson, A. G. H. and Sutherland, C. M. (1989). Doxorubicin-containing regimens for the treatment of stage II breast cancer: The National Surgical Adjuvant Breast and Bowel Project experience. Journal of Clinical Oncology 7: 572-582.

Fox, G., Popanda, O., Edler, L. and Thielmann, H. W. (1996). Preferential inhibition of DNA polymerases α, δ and ε from Novikoff hepatoma cells by inhibitors of cell proliferation. Journal of Cancer Research and Clinical Oncology 122: 78-94.

Fox, M. E and Smith, P. J. (1995). Subcellular localisation of the antitumour drug mitoxantrone and the induction of DNA damage in resistant and sensitive human colon carcinoma cells. Cancer Chemotherapy and Pharmacology 35: 403-410.

Fox, M. E and Smith, P. J. (1990). Long-term inhibition of DNA synthesis and the persistence

of trapped topoisomerase II complexes in determining the toxicity of the antitumour DNA intercalators m-AMSA and mitoxantrone. Cancer Research 50: 5813-5818.

Fox, K. R., Waring, M. J., Brown, J. R. and Neidle, S. (1986). DNA sequence preferences for the anticancer drug mitoxantrone and related anthraquinones revealed by DNase 1 footprinting. FEBS Letters 202: 289-294.

Foye, W. D., Vajragupta, O. and Sengupta, S. K. (1982). DNA-binding specificity and RNA polymerase inhibitory activity of bis(aminoalkyl)anthraquinones and bis (methyl thio)vinyl quinolinium iodides. Journal of Pharmaceutical Sciences 71: 253-257.

Frederick, C. A., Williams, L. D., Ughetto, G., van der Marel, G. A., Boom, J. H., Rich, A. and Wnag, A. H.-J. (1990). Structural comparison of anticancer drug-DNA complexes: adriamycin and daunomycin. Biochemistry 29: 2538-2549.

Friedman, O. M., Myles, A. and Colvin, M. (1979). Cyclphosphamide and phosphoramide mustards. Advances in Cancer Chemotherapy 1: 143-204.

Friedman, M. A., Bozdech, M. J., Billingham, M. E. and Rider, A. K. (1978). Doxorubicin cardiotoxicity. Serial endomyocardial biopsies and systolic time intervals. Journal of the American Medical Association 240: 1603-1606.

Fukushima, T., Ueda, T., Uchida, M. and Nakamura, T. (1993). Action mechanism of idarubicin (4-demethoxydaunorubicin) as compared with daunorubicin in leukemic cells. International Journal of Haematology 57: 121-130.

Gabizon, A. A. and Martin, F. (1997). Polyethylene-glycol-coated (pegylated) liposomal doxorubicin: rationale for use in solid tumours. Drugs 54 (suppl. 4): 30-35.

Gabizon, A. A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., Martin, F., Huang, A. and Barenholz, Y. (1994). Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Research 54: 987-992.

Gandecha, B. M., Brown, J. R. and Crampton, M. R. (1985). Dissociation kinetics of DNA-anthracycline and DNA-anthraquinone complexes determined by stop flow spectrophotometry. Biochemical Pharmacology 34: 733-736.

Gao, Y.-G. and Wang, A. H.-J. (1991). Influence of aglycone modifications on the binding of anthracycline drugs to DNA: the molecular structure of idarubicin and 4-*O*-demethyl-11-deoxydoxorubicin complexed to d(CGATCG). Anti-Cancer Drug Design 6: 137-149.

Gatto, B., Capranico, G. and Palumbo, M. (1999). Drugs acting on DNA topoisomerases: recent advances and future perspectives. Current Pharmaceutical design 5: 195-215.

Giaccone, G., Vanarkotte, J., Scagliotti, G., Capranico, G., Vandervalk, P., Rubio, G., Dalesio, O., Lopez, R., Zunino, F., Walboomers, J. and Pinedo, H. M. (1995). Differential expression of DNA topoisomerases in non-small-cell lung cancer and normal lung. Biochimica et Biophysica Acta 1264(3): 337-346.

Gibby, E. M., Boyse, O. and Hill, B. T. (1987). Selective interaction of verapamil with anthraquinones in adriamycin sensitive- and resistant- murine and human tumour cell lines *in vitro*. Cancer Chemotherapy and Pharmacology 20: 5-7.

Gibson, G. G. and Skett, P. (1986). Introduction to Drug Metabolism. Published by Chapman and Hall Limited, London.

Gibson, M. S. and Bradshaw, R. W. (1968). The Gabriel synthesis of primary amines. Angewandte Chemie (International Edition) 7: 919-930.

Goldstein, L. J., Galski, H., Fojo, A., Willingham, M. C., Lai, S-L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur G. M., Lieber, M., Cossman, J., Gottesman, M. M. and Pastan, I. (1989). Expression of a multidrug resistance gene in human cancers. Journal of the National Cancer Institute 81: 116-124.

Gollapudi, S., Thadepalli, F., Kim, C. H. and Gupta, S.(1995). Difloxacin reverses multidrug resistance in HL-60/AR cells that overexpress the multidrug resistance-related protein (MRP) gene. Oncology Research 7: 213-225.

Goodman, J. and Hochstein, P. (1977). Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. Biochemical and Biophysical Research Communications 77: 797-803.

Gottesman, M. M. and Pastan, I. (1993). Biochemistry of multi-drug resistance mediated by the multidrug transporter. Annual Review of Biochemistry 62: 385-427.

Graham, M. A., Clugson, C. K., King, L. H., Riley, R. J., Morrison, J. G., Cummings, J., Kerr, D. J. and Workman, P. (1992). Mechanistic studies with methoxymorpholinyl-doxorubicin: evidence of a novel covalently bound DNA adduct following activation by cytochrome P-450. Proceedings of the American Association Cancer Research 33: 513 (abstract 3065).

Graham, M. A., Newell, D. R., Butler, J., Hoey, B. and Patterson, L. H. (1987). The effect of the anthrapyrazole antitumour agent CI941 on rat liver microsome and cytochrome P-450 reductase mediated free radical processes. Biochemical Pharmacology 36(20): 3345-3351.

Gralla, J. D., Sasse-Dwight, S. and Poljak, L. G. (1987). Formation of blocking lesions at identical DNA sequences by the nitrosourea and platinum classes of anticancer drugs. Cancer Research 47: 5092-5096.

Grandi, M., Pezzoni, G., Ballinari, D., Capolongo, L., Suarato, A., Bargiotti, A., Faiardi, D. and Spreafico, F. (1990). Novel anthracycline analogues. Cancer Treatment Reviews 17: 133-138.

Grunicke, H., Hofmann, J., Utz, I. and Uberall, F. (1994). Role of protein kinases in antitumour drug-resistance. Annals of Hematology 69(1): S1-S6.

Guengerich, F. P. (1992). Characterization of human cytochrome P-450 enzymes. FASEB Journal 6: 745-748.

Haim, N., Nemec, J., Roman, J. and Sinha, B. K. (1987). *In vitro* metabolism of etoposide (VP-16-213) by liver microsomes and irreversible binding of reactive intermediates to microsomal proteins. Biochemical Pharmacology 36: 527-536.

Hammonds, T. R., Maxwell, A. and Jenkins, J. R. (1998). Use of a rapid throughput *in vivo* screen to investigate inhibitors of eukaryotic topoisomerase II enzymes. Antimicrobial Agents and Chemotherapy 42(4): 889-894.

Han, Y.-H., Austin, M. J. F., Pommier, Y. and Povirk, L. F. (1993). Small deletion and insertion mutations induced by the topoisomerase II inhibitor teniposide in CHO cells and comparison with sites of drug-stimulated DNA cleavage *in vitro*. Journal of Molecular Biology 229: 52-66.

Hanai, R., Caron, P. R. and Wang, J. C. (1996). Human TOP3: A single-copy gene encoding

DNA topoisomerase III. Proceedings of the National Academy of Sciences of the U.S.A. 93: 3653-3657.

Harker, W. G., Slade, D. L., Parr, R. L., Feldoff, P. W., Sullivan, D. M. and Holguin, M. H. (1995). Alterations in the topoisomerase IIα gene, messenger RNA, and subcellular protein distribution as well as reduced expression of the DNA topoisomerase IIβ enzyme in a mitoxantrone-resistant HL-60 human leukemia cell line. Cancer Research 55: 1707-1716.

Hellmann, K. (1996). Anthracycline cardiac toxicity prevention by dexrazoxane: breakthrough of a barrier- sharpens antitumour profile and therapeutic index (editorial). Journal of Clinical Oncology 1996: 14(2): 332-333.

Henderson, I. C., Allegra, J. C., Woodcock, T., Wolff, S., Brian, S., Cartwright, K, Dukart, G. and Henry, D. (1989). Randomized clinical trial comparing mitoxantrone with doxorubicin in previously treated patients with metastatic breast cancer. Journal of Clinical Oncology 7: 560-571.

Hertzberg, R. P., Busby, R. W., Caranfa, M. J., Holden, K G., Johnson, R. K., Hecht, S. M. and Kingsbury, W. D. (1990). Irreversible trapping of the DNA-topoisomerase I covalent complex. Journal of Biological Chemistry 265(31): 19287-19295.

Hickman, J. A (1992). Apoptosis induced by anticancer drugs. Cancer Metastasis Reviews 11: 121-139.

Higgins, C. F. (1992). ABC transporters from microorganisms to man. Annual Review of Cell Biology 8: 67-113.

Hill, B. T. (1996). Drug resistance: An overview of the current state of the art. International Journal of Oncology 9: 197-203.

Ho, C-K., Law, S-L., Chiang, H., Hsu, M-L., Wang, C-C. and Wang, S-Y. (1991). Inhibition of microtubule assembly is a possible mechanism of action of mitoxantrone. Biochemical and Biophysical Research Communications 180(1): 118-123.

Holden, J. A. (1997). Human deoxyribonucleic acid topoisomerases: Molecular targets of anticancer drugs. Annals of Clinical and Laboratory Science 27(6): 402-412.

Holley, R. W. (1975). Control of growth of mammalian cells in culture. Nature 258: 487-490.

Holm, C., Stearns, T. and Botstein, D. (1989). DNA topoisomerase II must act at mitosis to prevent nondisjunction and chromosome breakage. Molecular Cell Biology 9: 159-168.

Holmes, J. J. (1995). Metabolism and mechanisms of action of novel pyrazoloacridone and anthraquinone cytotoxic agents. Ph.D thesis, CRC, Glasgow.

Hortobágyi, G. N. (1997). Anthracyclines in the treatment of cancer. An overview. Drugs 54 (suppl. 4): 1-7.

Houlbrook, S., Addison, C. M., Davies, S. L., Carmichael, J., Stratford, I. J., Harris, A. L and Hickson, I. D. (1995). Relationship between expression of topoisomerase II isoforms and intrinsic sensitivity to topoisomerase II inhibitors in breast cancer cell lines. British Journal of Cancer 72: 1454-1461.

Hsiang, Y,-H. and Liu, L.F. (1989). Evidence for the reversibility of cellular DNA lesions induced by topoisomerase II poisons. Journal of Biological Chemistry 264: 9713-9715.

Hsiang, Y-H., Wu, H-Y. and Liu, L. F. (1988). Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. Cancer Research 48: 3230-3235.

Hu, X. F., Slater, A., Wall, D. M., Kantharidis, P., Parkin, J. D., Cowman, A. and Zalcberg, J. R. (1993). Rapid up-regulation of *mdr*1 expression by anthracyclines in a classical multidrug-resistant cell line. British Journal of Cancer 71: 931-936.

Huet, F., Lechevallier, A., Pellet, M. and Conia, J. M. (1978). Wet silica gel; a convenient reagent for deacetalization. Synthesis: 63-65.

Islam, S. A., Neidle, S., Gandecha, B. M., Partridge, M., Patterson, L. H. and Brown, J. R. (1985). Comparative computer graphics and solution studies of the DNA interaction of substituted anthraquinones based on doxorubicin and mitoxantrone. Journal of Medicinal Chemistry 28: 857-864.

Izquierdo, M. A., van der Zee, A., Vermorken, J., van der Valk, P., Belien, J., Giaccone, G., Scheffer, G. L., Flens, M. J., Kennemans, J., Pinedo, H. M., Meijer, C. J. L. M., de Vries, E. G. E. and Scheper, R. J. (1995). The drug resistance-associated marker LRP predicts poor

response to platinum-alkylating chemotherapy and outcome in advanced ovarian carcinoma. Journal of the National Institiute 87: 1230-1237.

Jameela, S. R., Latha, P. G., Subramonium, A. and Jayakrishnan, A. (1996). Antitumour activity of mitoxantrone-loaded chitosan microspheres against Ehrlich ascites carcinoma. Journal of Pharmacy and Pharmacology 48: 685-688.

Jarvinen, T. A. H., Kononen, J., Peltohuikko, M. and Isola, J. (1996). Expression of topoisomerase II-alpha is associated with rapid cell proliferation, aneuploidy and c-erbB-2 overexpression in breast cancer. American Journal of Pathology 148: 2073-2082.

Jenkins, J. R., Ayton, P., Jones, T., Davies, S. L., Simmons, D. L., Harris, A. L., Sheer, D. and Hickson, I. D. (1992). Isolation of cDNA clones encoding the β isoenzyme of human DNA topoisomerase II and localisation of the gene to chromosome 3p24. Nucleic Acids Research 20: 5587-5592.

Jenkins, T. C. (1989). Hypoxia-selective agents: radiosensitisers and cytotoxins. In; The Chemistry of Antitumour Agents. Ed. D. E. V. Wilman. Published by Blackie and Son, Glasgow.

Jensen, J. L. Lenz, P. A., Trusty, S., Sergi, V., Bell, K. and Rodgers, P. J. (1979). Substituent effects on the general- and specific-acid catalyzed hydrolyses of acetals and benzaldehydes. Journal of the American Chemical Society 101: 4672-4677.

Jesson, M. I., Johnston, J. B., Robotham, E. and Begleiter A. (1989). Characterization of the DNA-DNA cross-linking activity of 3'-(cyano-4-morpholinyl)-deamino adriamycin. Cancer Research 49: 7031-7036.

Jesson, M. I., Johnston, J. B., Anhalt, C. D. and Begleiter A. (1987). Effects of 3'-(3-cyano-4-morpholinyl)-3'-deamino adriamycin and structural analogues on DNA in HT-29 human colon carcinoma cells. Cancer Research 47: 5935-5938.

Johnson, R. K., Zee-Cheng, R. K-Y., Lee, W. W., Acton, E. M., Henry, D. W. and Cheng, C. C. (1979). Experimental antitumour activity of aminoanthraquinones. Cancer Treatment Reports 63(3): 425-439.

Jones, R. L., Lanier, A. C., Keel, R. A. and Wilson, W. D. (1980). The effect of ionic strength

on DNA-ligand unwinding angles for acridine and quinoline derivatives. Nucleic Acids Research 8: 1613-1624.

Juan, C.-C., Hwang, J., Liu, A.A., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M., Zhang, H., Wang, J. C. and Liu, L.F. (1988). Human topoisomerase I is encoded by a single copy gene that maps to chromosome region 20q12-13.2. Proceedings of the National Academy of Sciences of the U.S.A. 85: 8910-8913.

Kahles, H., Bastian, H. J., Schiffmann, O., Geck, M., Helmke, F. R. and Golz, N. (1997). Mitoxantrone-induced acute left heart failure after intrapleural instillation. Hertz 22(4): 217-220.

Kavallaris, M. (1997). The role of multidrug resistance-associated protein (MRP) expression in multidrug resistance. Anti-cancer Drugs 8(1): 17-25.

Kappus, H. (1986). Overview of enzyme systems involved in bioreduction of drugs and in redox cycling. Biochemical Pharmacology 35: 1-6.

Kapuscinski, J. and Darzynkiewicz, Z. (1986). Relationship between the pharmacological activity of antitumour drugs ametantrone and mitoxantrone (Novantrone) and their ability to condense nucleic acids. Proceedings of the National Academy of Sciences of the U.S.A. 83: 6302-6306.

Kapuscinski, J. and Darzynkiewicz, Z. (1985). Interactions of antitumour agents ametantrone and mitoxantrone (Novantrone) with double-stranded DNA. Biochemical Pharmacology 34(24): 4203-4213.

Kapuscinski, J., Darzynkiewicz, Z., Traganos, F. and Melamed, M. R. (1981). Interactions of a new antitumour agent, 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-9,10-anthracenedione, with nucleic acids. Biochemical Pharmacology 30: 231-240.

Kasahara, K., Fujiwara, Y., Sugimoto, Y., Nishio, K., Tamura, T., Matsuda, T. and Saijo, N. (1992). Determinants of response to the DNA topoisomerase II inhibitors doxorubicin and etoposide in human lung cancer cell lines. Journal of the National Cancer Institute 84: 113-118.

Kaufmann, S. H., Karp, J. E., Jones, R. J., Miller, C. B., Schneider, E., Zwelling, L. A., Cowan, K., Wendel, K. and Burke, P. J. (1994). Topoisomerase II levels and drug sensitivity in adult

acute myelogenous leukemia. Blood 83: 517-530.

Kawada, S-z., Yamashita, Y., Fujii, N. and Nakano, H. (1991). Induction of a heat stable topoisomerase II-DNA cleavable complex by nonintercalative terpenoids, Terpentecin and Clerocidin. Cancer Research 51: 2922-2925.

Keyes, S. R., Fracasso, P. M., Heimbrook, D. C., Rockwell, S., Sligar, S. G. and Sartorelli, A. C. (1984). Role of NADPH cytochrome c reductase and DT-diaphorase in the biotransformation of Mitomycin C. Cancer Research 44: 5638-5643.

Kharash, E. D. and Novak, R.F. (1985). Mitoxantrone and ametantrone inhibit hydroperoxide-dependent initiation and propagation reactions in fatty acid peroxidation. Journal of Biological Chemistry 260: 500-506.

Kharash, E. D. and Novak, R.F. (1983). Bis(alkylamino)-anthracenedione antineoplastic agent metabolic activation by NADPH-cytochrome P450 reductase and NADH dehydrogenase: diminished activity relative to anthracyclines. Archives of Biochemistry and Biophysics 224: 682-694.

Kharash, E. D. and Novak, R.F. (1982). Inhibition of adriamycin-stimulated microsomal lipid peroxidation by mitoxantrone and ametantrone, two new anthracenedione antineoplastic agents. Biochemical and Biophysical Research Communications 108: 1346-1352.

Khélifa, T., Casabianca-Pignède M.- R., René, B. and Jacquemin-Sablon, A. (1994). Expression of topoisomerase IIα and topoisomerase IIβ in Chinese hamster lung cells resistant to topoisomerase II inhibitors. Molecular Pharmacology 46(2): 323-328.

Kikuchi, M., Yamagishi, T. and Hida, M. (1982). Kinetic studies on the amination of leucoquinizarin. Bulletin of the Chemical Society of Japan 55: 1209-1212.

Kikuchi, M., Yamagishi, T. and Hida, M. (1981). The structure determination of leucoanthraquinones by proton and carbon-13 nuclear magnetic resonance spectroscopy. Dyes and Pigments 2: 143-151.

Kimura, K., Saijo, M., Ui, M. and Enomoto, T. (1994). Growth state-dependent and cell cycle-dependent fluctuation in the expression of two forms of topoisomerase II and possible specific modification of the higher molecular weight form in the M-phase. Journal of Biological

Chemistry 269(2): 1173-1176.

King, R. J. B. (1996). Cancer Biology. Published by Addison Wesley Longman Limited, Harlow, Essex.

Kohn, K. W., Hartley, J. A and Mattes, W. B (1987). Mechanisms of DNA sequence selective alkylation of G-N7 positions by nitrogen mustards. Nucleic Acids Research 15(24): 10531-10549.

Kohn, K. W., Spears, C. L. and Doty, P. (1966). Inter-strand crosslinking of DNA by nitrogen mustard. Journal of Molecular Biology 19: 266-288.

Kolodziejczyk, P., Reszka, K. and Lown, J. W. (1988). Enzymatic oxidative activation and transformation of the antitumour agent mitoxantrone. Free Radical Biology and Medicine 5: 13-25.

Krapcho, A. P., Getuhun, Z., Avery, K. L., Vargas, K. J., Hacker, M. P., Spinelli, S., Pezzoni, G. and Manzotti, C. (1991). Synthesis and antitumour evaluations of symmetrically and unsymmetrically substituted 1,4-bis[(aminoalkyl)amino]anthracene-9,10-diones and 1,4-bis[(aminoalkyl)amino]-5,8-dihydroxyanthracene-9,10-diones. Journal of Medicinal Chemistry 34: 2373-2380.

Krapcho, P. A., Menta, E., Oliva, A., Di Domenico, R. et al (1998). Synthesis and antitumour evaluation of 2,5-disubstituted-indazolo[4,3-gh]isoquinolin-6(2H)-ones (9-aza-anthrapyrazoles). Journal of medicinal chemistry 41: 5429-5444.

Krishnamoorthy, C. R., Yen, S-F., Smith, J. C., Lown, J. W. and Wilson, W. D. (1986). Stopped-flow kinetic analysis of the interaction of anthraquinone antitumour agents with calf thymus DNA, poly[d(G-C)]poly[d(G-C)] and poly[d(A-T)]poly[d(A-T)]. Biochemistry 25: 5933-5940.

Kronbach, T., Fischer, V. and Meyer, U. A. (1988). Cyclosporine metabolism in human liver: identification of a cytochrome P-450III gene family as the major cyclosporine-metabolizing enzyme explains interactions of cyclosporine with other drugs. Clinical Pharmacology and Therapeutics 43: 630-635.

Kubo, A., Nakagawa, K., Fukuoka, M., Yoshikawa, A., Harashima, T., Tamura, K., Yana, T.,

Masuda, N., Matsui, K., Kusunoki, Y., Kawase, I. and Takada, M. (1995). Identification of point mutations in the alpha topoisomerase II cDNA from human small cell lung cancer treated previously with etoposide. Proceedings of the American Association for Cancer Research 36: abstract 2664.

Kuss, B. J., Deeley, R. G., Cole, S. P. C., Willman, C. L., Kopecky, K. J. Wolman, S. R., Eyre, H. J., Lane, S. A., Nancarrow, J. K., Whitmore, S. A. and Callen, D. F. (1994). Deletion of the gene for multidrug resistance in acute myeloid leukaemia with inversion in chromosome 16: prognostic implications. Lancet 343: 1531-1534.

Lau, D. H. M., Duran, G. E., Lewis, A. D. and Sikic, B. I. (1994a). Metabolic conversion of methoxymorpholinyl doxorubicin: from a DNA strand breaker to a DNA cross-linker. British Journal of Cancer 70: 79-84.

Lau, D. H. M., Duran, G. E. and Sikic, B. I. (1994b). Analysis of intracellular retention of morpholinyl anthracyclines in multidrug resistant cancer cells by interactive laser cytometry. International Journal of Oncology 5: 1273-1277.

Lau, D. H. M., Duran, G. E. and Sikic, B. I. (1992). Analysis of intracellular uptake of anthracyclines by laser cytometry. Proceedings of the American Association of Cancer Research 33: 468 (abstract 2799).

Lau, D. H. M., Lewis, A. D., Duran, G. E. and Sikic, B. I. (1991). The cellular and biochemical pharmacology of the methoxy morpholino derivative of doxorubicin, FCE 23762. Proceedings of the National Academy of Sciences of the U.S.A. 32: 332 (abstract 1970).

Lau, D. H. M., Lewis, A. D. and Sikic, B. I. (1989). Association of DNA cross-linking with potentiation of the morpholino derivative of doxorubicin by human liver microsomes. Journal of the National Cancer Institute 81: 1034-1038.

Lawley, P. D. and Brookes, P. (1967). Interstrand cross-linking of DNA by difunctional alkylating agents. Journal of Molecular Biology 25: 143-160.

Lebsanft, J., McMahon, J. B., Steinmann, G. G. and Shoemaker, R. H. (1989). A rapid *in vitro* method for the evaluation of potential antitumour drugs requiring metabolic activation by hepatic S9 enzymes. Biochemical Pharmacology 38(24): 4477-4483.

Lee, J. S., Scala, S., Matsumoto, Y., Dickstein, B., Robey, R., Zhan, Z. R., Altenberg, G. and Bates, S. E. (1997). Reduced drug accumulation and multidrug resistance in human breast cancer cells without associated P-glycoprotein or MRP overexpression. Journal of Cellular Biochemstry 65(4): 513-526.

Legha, S., Benjamin, R. S., Mackay, B., Ewer, M., Wallace, S., Valdivieso, M., Rasmussen, S. L., Blumenschein, G. R. and Frierich, E. J. (1982). Reduction of doxorubicin cardiotoxicity by prolonged continuous IV infusion. Annals of Internal Medicine 96: 133-139.

Lerman, L. S. (1961). Structural considerations in the interaction of DNA and acridines. Journal of Molecular Biology 3: 18-30.

Leteurtre, F., Kohlhagen, G., Paull, K. D. and Pommier, Y. (1994). Topoisomerase II inhibition and cytotoxicity of the anthrapyrazoles DuP 937 and DuP 041 (Losoxantrone) in the National Cancer Institute preclinical antitumour drug discovery screen. Journal of the National Cancer Institute 86(16): 1239-1244.

Lewis, C. (1994). A review of the use of chemoprotectants in cancer chemotherapy. Drug Safety 11(3): 153-162.

Lewis, A. D., Lau, D. H. M., Duran, G. E., Wolf, C. R. and Sikic, B. I. (1992). Role of cytochrome P-450 from the human CYP3A gene family in the potentiation of morpholino doxorubicin by human liver microsomes. Cancer Research 52: 4379-4384.

Li, W. and Wang, J. C. (1998). Mammalian DNA topoisomerase II alpha is essential in early embryogenesis. Proceedings of the National Academy of Sciences of the U.S.A. 95(3): 1010-1013.

Ling, Y.-H., Priebe, W. and Perez-Soler, R. (1993). Apoptosis induced by anthracycline antibiotics in P388 in parent and multidrug resistant cells. Cancer Research 53: 1845-1852.

List, A. F., Spier, C. S., Grogan, T. M., Johnson, C., Roe, D. J., Greer, J. P., Wolff, S. N., Broxterman, H. J., Scheffer, G. L., Scheper, R. J. and Dalton, W. S. (1996). Overexpression of the major vault transporter protein lung-resistance protein predicts treatment outcome in acute myeloid leukemia. Blood 87(6): 2464-2469.

Locher, S. and Meyn, R (1983). Relationship between cytotoxicity and DNA damage in

mammalian cells treated with anthracenedione derivatives. Chemico-Biological Interactions 46: 369-379.

Lown, J. W. (1993). Anthracycline and anthraquinone anticancer agents: current status and recent developments. Pharmacology and Therapeutics 60(2): 185-214.

Lown, J. W. and Hanstock, C. C. (1985). High field ^{1}H-NMR analysis of the 1:1 intercalation complex of the antitumour agent mitoxantrone and the DNA duplex d[(CpGCpG)]$_2$. Journal of Biomolecular Structure and Dynamics 2: 1097-1106.

Lown, J. W., Morgan, A. R., Yen, S-F., Wang, Y-H. and Wilson, W. D. (1985). Characteristics of the binding of the anticancer agents mitoxantrone and ametantrone and related structures to deoxyribonucleic acids. Biochemistry 24: 4028-4035.

Lown, J. W., Hanstock, C. C., Bradley, R. D. and Scraba, D. G. (1984). Interactions of the antitumour agents mitoxantrone and bisantrene with deoxyribonucleic acids studied by electron microscopy. Molecular Pharmacology 25: 178-184.

Luch, A., Coffing, S. L., Tang, Y. M., Schneider, A., Soballa, V., Greim, H., Jefcoate, C. R., Seidel, A., Greenlee, W. F., Baird, W. M. and Doehmer, J. (1998). Stable expression of human cytochrome P-450 1B1 in V79 Chinese hamster cells and metabolically catalyzed DNA adduct formation of dibenzo[*a,l*]pyrene. Chemical Research in Toxicology 11: 686-695.

Lutzky, J., Astor, M. B., Taub, R. N., Baker, M. A., Bhalla, K., Gervasoni Jr.J. E., Rosado, M., Stewart, V., Krishna, S. and Hindenburg, A. A. (1989). Role of glutathione and dependent enzymes in anthracycline resistant HL-60/AR cells. Cancer Research 49: 4120-4125.

Malhotra, D. and Hopfinger, A. L. (1980). Conformational flexibility of dinucleotide dimers during unwinding from the B-form to an intercalation structure. Nucleic Acids Research 8: 5289-5300.

March, J. Advanced Organic Chemistry. Third edition. Published by J. Wiley and Sons, Inc., U.S.A.

Marinello, A. J., Bansal, S. K., Paul, B., Koser, P. L., Love, J., Struck, R. F. and Gurtoo, H. L. (1984). Metabolism and binding of cyclophosphamide and its metabolite acrolein to rat hepatic microsomal cytochrome P-450. Cancer Research 44: 4615-4621.

Mariani, M., Capolongo, L., Suarato, A., Bargiotti, A., Mongelli, N., Grandi, M. and Beck, W. T. (1994). Growth-inhibitory properties of novel anthracyclines in human leukemic cell lines expressing either P-gp-MDR or at-MDR. Investigation New Drugs 12(2): 93-97.

Marini, J. C., Miller, K. G. and Englund, P. T. (1980). Decatenation of kinetoplast DNA by topoisomerases. Journal of Biological Chemistry 255: 4976-4979.

Marmur, J. and Doty, P. (1959). Dependence on composition of the configurational stability of deoxyribonucleic acids. Nature 183: 1427-1429.

Masquellier, M., Baurain, R. and Trouet, A. (1980). Amino acid and dipeptide derivatives of daunorubicin. Synthesis, physicochemical properties, and lysozomal digestion. Journal of Medicinal Chemistry 23: 1166-1170.

Maxwell, A. and Gellert, M. (1986). Mechanistic aspects of DNA topoisomerases. Advances in Protein Chemistry 38: 69-107.

Mazerski, J., Martelli, S. and Borowski, E. (1998). The geometry of intercalation complex of antitumour mitoxantrone and ametantrone with DNA: molecular dynamics simulations. Acta Biochimica Polonica 45(1): 1-11.

Mazurek, C., Dutcher, J. P., Schwartz, E. L., Garl, S., Benson, L. and Wiernik, P. H. (1993). Phase I clinical trial and pharmacokinetic study of menogaril (7-con-*O*-methylnogarol) in previously treated patients with acute leukaemia. Investigational New Drugs 11: 313-322.

McClean, S. and Hill, B. T. (1993). Evidence of post-translational regulation of P-glycoprotein associated with the expression of a distinctive multiple drug resistance phenotype in Chinese hamster ovary cells. European Journal of Cancer 29: 2243-2248.

McGhee, J. D. and von Hippel, P. H. (1974). Theoretical aspects of DNA-protein interactions: co-operative and non-co-operative binding of large ligands to a one-dimensional homogeneous lattice. Journal of Molecular Biology 86: 469-489.

McKay, J. A. Murray, G. I., Weaver, R. J., Ewen, S. W. B., Melvin, W. T. and Burke, M. D. (1993). Xenobiotic metabolizing enzyme expression in colonic neoplasia. Gut 34: 1234-1239.

McKeown, S. R., Friery O. P., McIntyre, I. A., Hejmadi, M. V., Patterson, L. H. and Hirst, D.

G. (1996). Evidence for a therapeutic gain when AQ4N or tirapazamine is combined with radiation. British Journal of Cancer 74(S27): S39-S42.

McKeown, S. R., Hejmadi, M. V., McIntyre, I. A., McAleer, J. J. A. and Patterson, L. H. (1995). AQ4N- an alkylaminoanthraquinone N-oxide showing bioreductive potential and positive interaction with radiation *in vivo*. British Journal of Cancer 72(1): 76-81.

Meriweather, V. D. and Bachur, N. R (1972). Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukaemia. Cancer Research 32: 1137-1142.

Mewes, K., Blanz, J., Ehninger, G., Gebhardt, R. and Zeller, K-P. (1993). Cytochrome P-450-induced cytotoxicity of mitoxantrone by formation of electrophilic intermediates. Cancer Research 53: 5135-5142.

Miller, J. C. and Miller, J. N. (1993). Statistics for Analytical Chemistry (third edition). Publ. Ellis Horwood.

Miller, T. P., Grogan, T. M., Dalton, E., Spier, C. M., Scheper, R. J. and Salmon, S. E. (1991). P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high dose verapamil. Journal of Clinical Oncology 9: 17-24.

Miller, K. G., Liu, L. F. and Englund, P. T. (1981). A homogeneous type II DNA topoisomerase from HeLa cell nuclei. Journal of Biological Chemistry 256: 9334-9339.

Mimnaugh, E. G., Trush, M. A., Bhatnagar, M. and Gram, T. E. (1985). Enhancement of reactive oxygen-dependent mitochondrial membrane lipid peroxidation by the anticancer drug adriamycin. Biochemical Pharmacology 34: 847-856.

Minderman, H., Linssen, P., van der Lely, N., Wessels, J., Boezeman, J., De Witte, T. and Haanen, C. (1994). Toxicity of idarubicin and doxorubicin towards normal and leukemic human bone marrow progenitors in relation to their proliferative state. Leukemia 8: 382-387.

Mirski, S. E. L., Evans, C. D., Almquist, K. C., Slovak, M. L. and Cole, S. P. C. (1993). An altered topoisomerase IIα in a drug-resistant small cell lung cancer cell line selected in VP-16. Cancer Research 53: 4866-4873.

Monnot, M., Mauffret, O., Simon, V., Lescot, E., Psaume, B., Saucier, J-M., Charra, M.,

Belehradek, J. and Fermandjian, S. (1991). DNA-drug recognition and effects on topoisomerase II-mediated cytotoxicity. Journal of Biological Chemistry 266(3): 1820-1829.

Morris, J.G. (1985). A Biologist's Physical Chemistry. Ed. E. J. W. Barrington and A. J. Willis. Published by Edward Arnold Limited, London.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65: 55-63.

Muggia, F. M. (1997). Clinical efficacy and prospects for use of pegylated liposomal doxorubicin in the treatment of ovarian and breast cancers. Drugs 54 (suppl. 4): 22-29.

Muggia, F. M., Dimery, I and Arbuck, S. G. (1996). Camptothecin and its analogs- An overview of their potential in cancer therapeutics. Annals of the New York Academy of Sciences 803: 213-223.

Muindi, J., Sinha, B. K., Gianni, L. and Myers, C. (1985). Thiol-dependent DNA damage produced by anthracycline-iron complexes: the structure-activity relationships and molecular mechanisms. Molecular Pharmacology 27: 356-365.

Murdock, K. C., Child, R. G., Fabio, P. F., Angier, R. B., Wallace, R. E., Durr, F. E. Citarella, R. V. (1979). Antitumour agents. 1. 1,4-bis[(aminoalkyl)amino]-9,10-anthracenediones. Journal of Medicinal Chemistry 22(9): 1024-1030.

Murphy, D. S., McHardy, P., Coutts, J., Mallon, E. A., George, W. D., Kaye, S. B., Brown, R. and Keith, W. N. (1995). Interphase cytogenetic analysis of erbB2 and topo IIα co-amplification in invasive breast cancer and polysomy of chromosome 17 in ductal carcinoma *in situ*. International Journal of Cancer 64: 18-26.

Murray, G. I., Taylor, V. E., McFaden, M. C. E., McKay, J. A., Greenlee, F. W., Burke, M. D., and Melvin, W. T. (1997). Tumour-specific expression of cytochrome P450 CYP1B1. Cancer Research 57: 3026-3031.

Murray, G. I., Taylor, V. E., McKay, J. A., Weaver, R. J., Ewen, S. W. B., Melvin, W. T. and Burke, M. D. (1995). Expression of xenobiotic metabolizing enzymes in tumours of the urinary bladder. International Journal of Experimental Pathology 76: 271-276.

Murray, G. I., Shaw, D., Weaver, R. J., McKay, J. A., Ewen, S. W. B., Melvin, W. T. and Burke, M. D. (1994). Cytochrome P450 expression in oesophageal cancer. Gut 35: 599-603.

Negri, C., Chiesa, R., Cerino, A., Bestagno, M., Sala, C., Zini, N., Maraldi, N. M. and Astaldi Ricotti, G. C. B. (1992). Monoclonal antibodies to human DNA topoisomerase I and the two isoforms of DNA topoisomerase II: 170- and 180-kDa isozymes. Experimental Cell Research 200: 452-459.

Nelson, R., Kamataki, T., Waxman, D., Guengerich, F., Estabrook, R., Feyereisen, R., Gonzalez, F., Coon, M., Gunsalus, I., Gotoh, O., Okuda, K. and Nebert, D. (1993). The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. DNA and Cell Biology 12: 1-51.

Nelson, E. M., Tewey, K. M. and Liu, L. F. (1984). Mechanism of antitumor drugs. Poisoning of mammalian DNA topoisomerase II on DNA by an antitumor drug *m*-AMSA. Proceedings of the National Academy of Sciences of the U.S.A. 81: 1361-1365.

Nguyen, B and Gutierrez, P. L. (1990). Mechanism(s) for the metabolism of mitoxantrone: electron spin resonance and electrochemical studies. Chemico-biological Interactions 74: 139-162.

Nooter, K. and Stoter, G. (1996). Molecular mechanisms of multidrug resistance in cancer chemotherapy. Pathology Research and Practice 192: 786-780.

Nordsmark, M., Bentzen, S. M. and Overgaard, J. (1994). Measurement of human tumour oxygenation status by a polarographic needle electrode. Acta Oncologica 33: 383-389.

Ohno, Y. and Ormstad, K. (1985). Formation, toxicity and inactivation of acrolein during biotransformation of cyclophosphamide as studied in freshly isolated cells from rat liver and kidney. Archives in Toxicology 57: 99-103.

Osheroff, N. (1987). Role of the divalent cation in topoisomerase II mediated reactions. Biochemistry 26: 6402-6406.

Osheroff, N. (1986). Eukaryotic topoisomerase II: Characterization of enzyme turnover. Journal of Biological Chemistry 261: 9944-9950.

Ota, E., Abe, Y., Oshika, Y., Ozeki, Y., Iwasaki, M., Inoue, H., Yamazaki, H., Ueyama, Y., Takagi, K., Ogata, T., Tamaoki, N. and Nakamura, M (1995). Expression of the multidrug resistance-associated protein (MRP) gene in non-small-cell lung cancer. British Journal of Cancer 72: 550-554.

Ozols, R. F. (1995). Clinical reversal of drug resistance. Current Problems in Cancer 19(2): 69-123.

Pachter, J. A., Huang, C.-H., Du Vernay, V. H., Prestayko, A. W. and Crooke, S. T. (1982). Viscometric and fluorometric studies of deoxyribonucleic acid interactions of several new anthracyclines. Biochemistry 21: 1541-1547.

Panousis, C., Kettle, A. J. and Phillips, D. R. (1997). Neutophil-mediated activation of mitoxantrone to metabolites which form adducts with DNA. Cancer Letters 113(1-2): 173-178.

Panousis, C., Kettle, A. J. and Phillips, D. R. (1995). Myeloperoxidase oxidises mitoxantrone to metabolites which bind covalently to DNA and RNA. Anti-cancer Drug Design 10(8): 593-605.

Panousis, C., Kettle, A. J. and Phillips, D. R. (1994). Oxidative metabolism of mitoxantrone by the human neutophil enzyme myeloperoxidase. Biochemical Pharmacology 48(12): 2223-2230.

Panousis, C. and Phillips, D. R. (1994). DNA sequence specificity of mitoxantrone. Nucleic Acids Research 22(8): 1342-1345.

Papahadjopoulos, D., Allen, T. M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S. K., Lee, K. D., Woodle, M. C., Lasic, D. D., Redemann, C. and Martin, F. J. (1991). Sterically stabilized liposomes: improvements in pharmacokinetics and therapeutic efficacy. Proceedings of the National Academy of Sciences of the U.S.A. 88: 11460-11464.

Park, J. G., Kramer, B. S., Steinberg, S. M., Carmichael, J., Collins, J. M., Minna, J. and Gazdar, A. F. (1987). Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. Cancer Research 47: 5875-5879.

Patel, D. J. and Canuel, L. L. (1978). Anthracycline antitumor antibiotic. nucleic acid interactions. European Journal of Biochemistry 90: 247-254.

Patterson, L. H. and Raleigh, S. M. (1998). Reductive metabolism: its application in prodrug

activation. In: Drug Metabolism: Towards the Next Millenium. Biomedical and Health Research 25: 72-79. Ed. N. Gooderham. Published by IOS Press.

Patterson, L. H., Craven, M. R., Fisher, G. R. and Teesdale-Spittle, P. (1994). Aliphatic amine N-oxides of DNA binding agents as bioreductive drugs. Oncology Research 6(10-11): 533-538.

Patterson, L. H. (1993). Rationale for the use of aliphatic N-oxides of cytotoxic anthraquinones as prodrug DNA binding agents: a new class of bioreductive agent. Cancer and metastasis reviews 12: 119-134.

Patterson, L. H., Maine, J. E., Cairns, D., Craven, M. R., Bennett, N., Fisher, G. R., Ruparelia, K. and Giles, Y. C. (1992). Use of N-oxides of DNA affinic anthraquinones as bioreductive prodrugs. Annals of Oncology 3 (suppl.): 94.

Pincus, R. and Goldman, D. (1990). Evidence for impaired mitoxantrone and vinblastine binding in P388 murine leukaemia cells with multidrug resistance. Biochemical Pharmacology 40: 2625-2635.

Pieper, R. O., Futscher, B. W. and Erickson, L. C. (1989). Transcription-terminating lesions induced by bifunctional alkylating agents *in vitro*. Carcinogenesis 10: 1307-1314.

Pietrusko, R., Kurys, G. and Ambroziak, W. (1991). Physiological role of aldehyde dehydrogenase (EC 1.2.1.3). Alcoholism 206: 101-106.

Pohle, W., Bohl, M., Flemming, J. and Bohlig, H. (1990). Subsidiary hydrogen bonding of intercalated anthraquinonic anticancer drugs to DNA phosphate. Biophysical Chemistry 35: 213-226.

Pommier, Y., Leteurtre, F., Fesen, M. R., Fujimori, A., Bertrand, R, Solary, E., Kohlhagen, G. and Kohn, K. W. (1994). Cellular determinants of sensitivity and resistance to DNA topoisomerase inhibitors. Cancer Investigation 12(5): 530-542.

Pommier, Y., Orr, A, A., Kohn, K. W., Riou, J-F. (1992). Differential effects of amsacrine and epipodophyllotoxins on topoisomerase II cleavage in the human c-*myc* proto-oncogene. Cancer Research 52: 3125-3130.

Posner, L. E., Dukart, G., Goldberg, J., Bernstein, T. and Cartwright, K. (1985). Mitoxantrone:

an overview of safety and toxicity. Investigational New Drugs 3: 123-132.

Potesmil, M., Hsiang, Y.-H., Liu, L. F., Bank, B., Grossberg, H., Kirschenbaum, S., Forlenzar, T. J., Penziner, A., Kanganis, D., Knowles, D., Traganos, F., Silber, R. (1988). Resistance of human leukemic and normal lymphocytes to drug-induced DNA cleavage and low levels of DNA topoisomerase II. Cancer Research 48: 3537-3543.

Prost, S. (1995). Mechanisms of resistance to topoisomerase poisons. General Pharmacology 26(8): 1773-1784.

Qiu, J., Catapano, C. V. and Fernandes, D. J. (1996). Formation of topoisomerase IIα complexes with nascent DNA is related to VM-26-induced cytotoxicity. Biochemistry 35: 16354-16360.

Rajagopalan, S., Politi, P. M., Sinha, B. K. and Myers, C. E. (1988). Adriamycin-induced free radical formation in the perfused rat heart: implications for cardiotoxicity. Cancer Research 48: 4766-4769.

Raleigh, S. M., Burke, M. D., Murray, G. I. and Patterson, L. H. (1996). Involvement of renal cell carcinoma cytochrome P-450 in bioreduction of AQ4N, a hypoxic tumour activated topoisomerase II inhibitor. Annals of Oncology 7 (suppl.): 81.

Randall, K., Broome, M. G., Hoard, W. S., Evans, S. F. and Pritchard, D. F. (1983). Experimental therapeutic and biochemical studies of anthracenedione derivatives. In: New Anticancer Drugs: Mitoxantrone and Bisantrene. pp1-28. Ed. M Rozencweig. Published by Raven Press, New York, U.S.A.

Rawn, J. D. (1983). Biochemistry. Ed. M. Wasserman. Published by Harper and Row Inc., New York, U.S.A.

Rehn, C. and Pindur, U. (1996). Model building and molecular mechanics calculations of mitoxantrone-deoxytetranucleotide complexes: Molecular foundations of DNA intercalation as cytostatic active principle. Monatshefte für Chemie 127: 631-644.

Reinert, K. E. (1983). Anthracycline-binding induced DNA stiffening, bending and elongation: stereochemical implications from viscometric investigations. Nucleic Acids Research 11: 3411-3440.

Rekha, G. K., Sreerama, L. and Sladek, N. E. (1994). Intrinsic cellular resistance to oxazaphosphorines exhibited by a human colon carcinoma cell line expressing relatively large amounts of a class-3 aldehyde dehydrogenase. Biochemical Pharmacology 48: 1943-1952.

Reszka, K. J., Matuszak, Z. and Chignell, C. F. (1997). Lactoperoxidase-catalysed oxidation of the anticancer agent mitoxantrone by nitrogen dioxide. Chemical Research in Toxicology 10(12): 1325-1330.

Reszka, K., Hartley, J. A., Kolodziejczyk, P. and Lown, J. W. (1989). Interaction of the peroxidase-derived metabolite of mitoxantrone with nucleic acids. Evidence for covalent binding of ^{14}C-labeled drug. Biochemical Pharmacology 38: 4253-4260.

Reszka, K., Kolodziejczyk, P. and Lown, J. W. (1986). Horseradish peroxidase-catalysed oxidation of mitoxantrone. Spectrophotometric and electron paramagnetic resonance studies. Free Radical Biology and Medicine 2: 25-32.

Richard, B., Fabre, G., De Sousa, G., Fabre, I., Rahmani, R. and Cano, J-P. (1991). Interspecies variability in mitoxantrone metabolism using primary cell cultures of hepatocytes isolated from rat, rabbit and humans. Biochemical Pharmacology 41(2): 255-262.

Richard, B., Fabre, G., Fabre, I. and Cano, J.-P. (1989). Excretion and metabolism of mitoxantrone in rabbits. Cancer Research 49: 833-837.

Riley, R. and P. Workman (1992). DT-diaphorase and cancer chemotherapy. Biochemical Pharmacology 43: 1657-1669.

Riou, J.-F., Lefevre, D. and Riou, G. (1989). Stimulation of the topoisomerase II induced cleavage sites in the c-*myc* protooncogene by antitumour drugs is associated with gene expression. Biochemistry 28: 9104-9110.

Ripamonti, M., Capolongo, L., Melegaro, G., Gornati, C., Bargiotti, A., Caruso, M., Grandi, M. and Suarato, A. (1996). Morpholinylanthracylines-cytotoxicity and antitumour activity of differently modified derivatives. Investigational New Drugs 14(2): 139-146.

Ripamonti, M., Pezzoni, G., Pesenti, E., Pastori, A., Farao, M., Bargiotti, A., Suarato, A., Spreafico, F. and Grandi, M. (1992). *In vivo* anti-tumour activity of FCE 23762, a methoxy-morpholinyl derivative of doxorubicin active on doxorubicin-resistant tumour cells. British

Journal of Cancer 65: 703-707.

Roberts, R. A., Cress, A. E. and Dalton, W. S. (1989). Persistent intracellular binding of mitoxantrone in a human colon carcinoma cell line. Biochemical Pharmacology 38(23): 4283-4290.

Roca, J., Berger, J. M., Harrison, S. C. and Wang, J. C. (1996). DNA transport by a type II topoisomerase: direct evidence for a two-gate mechanism. Proceedings of the National Academy of Sciences of the U.S.A. 93: 4057-4062.

Roca, J. and Wang, J. C. (1992). The capture of a DNA double helix by an ATP-dependent protein clamp: a key step in DNA transport by type two DNA topoisomerases. Cell 71: 833-840.

Rogan, A. M., Hamilton, T. C., Young, R. C., Klecker Jr, R. W. and Ozols, R. F. (1984). Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science 224: 994-996.

Safa, A.R. and Tseng, M. T. (1984). Inhibition of protein synthesis and cell proliferation in cultured human breast cancer cells treated with mitoxantrone. Cancer Letters 24: 317-326.

Safa, A.R., Chegini, N and Tseng, M. T. (1983). Influence of mitoxantrone on nucleic acid synthesis on the T-47D breast tumour cell line. Journal of Cellular Biochemistry 22: 111-120.

Sahai, B. M. and Kaplan, J. G. (1986). A quantitative decatenation assay for type II topoisomerases. Analytical Biochemistry 156: 364-379.

Sat, Y. N., Bibby, M. and Duncan, R. (1999). Effect of tumour size and tumour type on passive tumour accumulation of polymeric anticancer agents. British Journal of Cancer 81(4): 583 (abstract P18).

Schellens, J. H., Planting, A. S., van Acker, B. A., Loos, W. J., de Boer-Dennert, M., van der Burg, M. E., Koier, I., Krediet, R. T., Stoter, G. and Verweij, J. (1994). Phase I and pharmacologic study of the novel indoloquinone bioreductive alkylating cytotoxic drug EO9. Journal of the National Cancer Institute 86: 906-912.

Schleyer,, E., Kamischke, A., Kaufmann, C. C., Unterhalt, M. and Hiddemann, W. (1994). New

aspects on the pharmacokinetics of mitoxantrone and its two major metabolites. Leukaemia 8(3): 435-440.

Schmalix, W., Lang, D., Schneider, A., Bocker, R., Greim, H. and Doehmer. J. (1996). Stable expression and coexpression of human cytochrome P-450 oxidoreductase and cytochrome P-450 1A2 in V79 Chinese hamster cells- sensitivity to quinones and biotransformation of 7-alkoxyresorufins and triazines. Drug Metabolism and Disposition 24(12): 1314-1319.

Schneider, E., Lawson, P. A. and Ralph, R. K. (1989). Inhibition of protein synthesis reduces the cytotoxicity of 4'-(9-acridinylamino)-methanesulfon-*m*-anisidide without affecting DNA breakage and DNA topoisomerase II in a murine mastocytoma cell line. Biochemical Pharmacology 38: 263-269.

Schoenlein, P. V. (1993). Molecular cytogenetics of multiple drug resistance. Cytotechnology 12(1-3): 63-89.

Schott , B., Vrignaud. P., Ries, C., Robert, J. and Londos-Gagliardi, D. (1990). Cellular pharmacology of 4'-iodo-4'-deoxydoxorubicin. British Journal of Cancer 61: 543-547.

Shan, K., Lincoff, A. M. and Young, J. B. (1996). Anthracycline-induced cardiotoxicity. Annals of Internal medicine 125: 47-58.

Shenkenberg, T. D. and von Hoff, D. D. (1986). Mitoxantrone. A new anticancer drug with significant clinical activity. Annals of Internal Medicine 105: 67-81.

Shih, L. B., Goldenberg, D. M., Xuan, H., Lu, H., Sharkey, R. M. and Hall, T. (1991). Anthracycline conjugates prepared by a site-specific linkage via an amino-dextran intermediate carrier. Cancer Research 51: 4192-4198.

Showalter, H. D. H., Johnson, J. L., Hoftiezer, J. M., Turner, W. R., Werbel, L. M., Leopold, W. R., Shillis, J. L., Jackson, R. C. and Elslager, E. F. (1987). Anthrapyrazole anticancer agents. Synthesis and structure-activity relationships against murine leukaemias. Journal of Medicinal Chemistry 30: 121-131.

Sikic, B. I., Ehsan, M.N., Harker, W. G., Friend, N. F., Brown, B. W., Newman, R. A., Hacker, M. P. and Acton, E. M. (1985). Dissociation of antitumour potency from anthracycline cardiotoxicity in a doxorubicin analog. Science 228: 1544-1546.

Simon, M. S. (1963). Spectral shifts in anthraquinone dyes caused by non-conjugated substituents. Journal of the American Chemical Society 85: 1974-1977.

Sinha, B. K., Motten, A. G. and Hanck, K. W. (1983). The electrochemical reduction of 1,4-bis-[2-[(2-hydroxyethyl)-amino]-ethylamino]-anthracenedione and daunomycin: biochemical significance in superoxide formation. Chemico-Biological Interactions 43: 371-377.

Sladek, N. E. (1987). Oxazaphosphorines. In: Metabolism and Action of Anticancer Drugs, pp 48-90. Ed. G. Powys and R. A. Prough. Published by Taylor and Francis, New York.

Slusarska, E. and Zwierzak, A. (1981). One pot phase-transfer-catalysed *N*-alkylation of diphenylphosphinamide with alcohols in the presence of methanesulfonyl chloride. Synthesis: 155-156.

Smith, P. J., Desnoyers, R., Blunt, N., Giles, Y., Patterson, L. H. and Watson, J. V. (1997a). Flow cytometric analysis and confocal imaging of anticancer alkylaminoanthraquinones and their N-oxides in intact human cells using 647-nm krypton laser excitation. Cytometry 27: 42-53.

Smith, P. J., Blunt, N., Desnoyers, R., Giles, Y., Patterson, L. H. and Watson, J. V. (1997b). DNA topoisomerase II-dependent cytotoxicity of alkylaminoanthraquinones and their N-oxides. Cancer Chemotherapy and Pharmacology 39(5) 455-461.

Smith, P. J., Rackstraw, C. and Cotter, F (1994a). DNA fragmentation as a consequence of cell-cycle traverse in doxorubicin-treated and idarubicin-treated human lymphoma cells. Annals of Hematology 69(1): S7-S11.

Smith, C., Smith, G. and Wolf, C. (1994b). Genetic polymorphism in xenobiotic metabolism. European Journal of Cancer 30A: 1935-1941.

Smith, P. J., Sykes, H. R., Fox, M. E. and Furlong, I. J. (1992). Subcellular distribution of the anticancer drug mitoxantrone in human and drug-resistant murine cells analysed by flow cytometry and confocal microscopy and its relationship to the induction of DNA damage. Cancer Research 52: 4000-4008.

Smith, P. J., Morgan, S. A., Fox, M. E. and Watson, J. V. (1990). Mitoxantrone-DNA binding and the induction of topoisomerase II associated DNA damage in multi-drug resistant small cell

lung cancer cells. Biochemical Pharmacology 40(9): 2069-2078.

Smith, P. J. and Makinson, T. A. (1989). Cellular consequences of overproduction of topoisomerase II in an ataxia-telangiectasia cell line. Cancer Research 49: 1118-1124.

Sonneveld, P., Durie, B. G. M., Lokhorst, H. M., Maric, T., Soebu, G., Sucin, S., Zittonn, R., Cowenberg, B., Nooter, K. (1992). Modulation of mulidrug-resistant multiple myeloma by cyclosporin. Lancet 340: 255-259.

Spatzenegger, M. and Jaeger, W. (1995). Clinical importance of hepatic cytochrome P450 in drug metabolism. Drug Metabolism Reviews 27(3): 397-417.

Stammler, G., Sauerbrey, A. and Volm, M. (1994). Determination of DNA topoisomerase II in newly diagnosed childhood leukemia by immunocytochemistry and RT-PCR. Cancer Letters 84: 141-147.

Stilmer, F. and Perkins, M. (1955). The Chemistry of Synthetic Dyes and Pigments. Ed. H. Lubs, New York, U. S. A.

Streeter, D. G., Johl, J. S., Gordon, G. R. and Peters, J. H. (1986). Uptake and retention of morpholinyl anthracyclines by adriamycin-sensitive and -resistant P388 cells. Cancer Chemotherapy and Pharmacology 16: 247-252.

Suarato, A., Angelucci, F., Bargiotti, A., Caruso, M., Faiardi, D., Capolongo, L., Geroni, C., Ripamonti, M. and Grandi, M. (1995). Synthesis and study of structure-activity relationships of new classes of anthracyclines. ACS Symposium Series 574: 142-155.

Sullivan, D. M., Latham, M. D. and Ross, W. E. (1987). Proliferation-dependent topoisomerase II content as a determinant of antineoplastic drug action in human, mouse and Chinese hamster ovary cells. Cancer Research 47: 3973-3979.

Supino, R., Necco, A., Dasdia, T , Casazza, A. M. and Di Marco, A. (1977). Relationship between effects on nucleic acid synthesis in cell cultures and cytotoxicity of 4-demethoxy derivatives of daunorubicin and adriamycin. Cancer Research 37: 4523-4528.

Suzuki, H., Ikeda, T., Yamagishi, T., Nakaike, S., Nakane, S. and Ohsawa, M. (1995). Efficient induction of chromosome-type aberrations by topoisomerase II inhibitors closely associated

with stabilization of the cleavable complex in cultured fibroblast cells. Mutation Research 328(2): 151-161.

Suzuki, H. and Nakane, S. (1994). Differential induction of chromosomal aberrations by topoisomerase inhibitors in cultured Chinese hamster cells. Biological and Pharmaceutical Bulletin 17(2): 222-226.

Szeja, (1979). Synthesis of sulfonic esters under phase-transfer catalysed conditions. Synthesis: 822.

Talbot, D. C., Smith, I. E., Mansi, J. L., Hudson, I., Calvert, A. H. and Ashley, S. E. (1991). Anthrapyrazole CI941: A highly active new agent in the treatment of advanced breast cancer. Journal of Clinical Oncology 9: 2141-2147.

Tannock, I. F. (1968). The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. British Journal of Cancer 22: 258-273.

Teicher, B. A., Holden, S. A., Al-Achi, A. and Herman, T. S. (1990). Classification of antineoplastic treatments by their differential toxicity toward putative oxygenated and hypoxic tumor subpopulations *in vivo* in the FSaIIC murine fibrosarcoma. Cancer Research 50: 3339-3344.

Teicher, B. A., Lazo, J. S. and Sartorelli, A. C. (1981). Classification of antineoplastic agents by their selective toxicities toward oxygenated and hypoxic tumor cells. Cancer Research 41: 73-81.

Testi, R., Mattii, L., Disimone, D., Zaccaro, I., Malvaldi, G., Grassi, B and Petrini, M. (1995). Evaluation of resistance index of several anticancer agents on parental and resistant P-388 cell lines. Leukemia Research 19(4): 257-261.

Tewey, K. M., Rowe, T. C., Yang, L., Halligan, B. D. and Liu, L. F. (1984a). Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466-468.

Tewey, K. M., Chen, G. L., Nelson, E.M. and Liu, L. F. (1984b). Intercalative antitumour drugs interfer with the breakage-reunion reaction of mammalian DNA topoisomerase II. Journal of Biological Chemistry 259: 9182-9187.

Thiebaut , F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1987). Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. Proceedings of the National Academy of Sciences of the U.S.A. 84: 7735-7738.

Tholimson, R. H. and Gray, L. H. (1955). The histological structure of some human lung cancers and the possible implications for radiotherapy. British Journal of Cancer 9: 539-549.

Thomas, C. B., Kohn, K. W. and Bonner, W. B. (1978). Characterization of DNA-protein cross-links formed by treatment of L1210 cells and nuclei with *bis*(2-chloroethyl)methylamine (nitrogen mustard). Biochemistry 17: 3954-3958.

Tomasz, M. and Palom, Y. (1997). The mitomycin bioreductive antitumour agents: Cross-linking and alkylation of DNA as the molecular basis of their activity. Pharmacology and Therapeutics 76(1-3): 73-87.

Tong, G. L., Henry, D. W. and Acton, E. M. (1979). 5-iminodaunorubicin. Reduced cardiotoxic properties in an antitumour anthracycline. Journal of Medicinal Chemistry 22: 36-39.

Torti, F. M., Bristow, M. R., Howes, A. E., Aston, D., Stockdale, F. E., Carter, S. K., Kohler, M., Brown Jr, B. and Billingham, M. E. (1983). Reduced cardiotoxicity of doxorubicin delivered on a weekly schedule. Annals of Internal Medicine 99: 745-749.

Toussaint, C., Albin, N., Maassad, L., Grunenwald, D., Parise, O., Morizet, J., Gouyette, A. and Chabot, G. (1993). Main drug- and carcinogen-metabolizing enzyme systems in human non-small cell lung cancer and peritumoral tissues. Cancer Research 53: 4608-4612.

Towatari, M., Ito, Y., Morishita, Y., Tanimoto, M., Kawashima, K., Morishima, Y., Andoh, T. and Saito, H. (1990). Enhanced expression of DNA topoisomerase II by recombinant human granulocyte colony-stimulating factor in human leukaemia cells. Cancer Research 50: 7198-7202.

Traganos, F., Evenson, D. P., Staiano-Coico, L., Darzynkiewicz, Z. and Melamed, M. R. (1980). Action of dihydroxyanthraquinone on cell cycle progression and survival of a variety of cultured mammalian cells. Cancer Research 40: 671-681.

Tritton, T. R. (1991). Cell death in cancer chemotherapy: the case of adriamycin. In: Apoptosis: the Molecular Basis of Cell Death. Current Communications in Cell and Molecular Biology 3.

Ed. L. D. Tomei and F. O. Cope. Published by Cold Spring Harbor Laboratory Press, New York, U. S. A.

Tritton, T. R. and Yee, G. (1982). The anticancer drug adriamycin can be actively cytotoxic without entering cells. Science 217: 248-250.

Trouet, A., Masquellier, M., Baurain, R., and Campeneere, D. (1982). A covalent linkage between daunorubicin and proteins that is stable in serum and reversible by lysosomal hydrolases, as required for a lysosomotropic drug-carrier conjugate. *In vitro* and *in vivo* studies. Proceedings of the National Academy of Sciences of the U.S.A. 79: 626-629.

Tsai-Pflugfelder, M. L., Liu, L. F., Liu, A. A., Tewey, K. M., Whang-Peng, J., Knutsen, T., Huebner, K., Croce, C. M. and Wang, J. C. (1988). Cloning and sequencing of cDNA encoding human topoisomerase II and localization of the gene to chromosome region 17q21-22. Proceedings of the National Academy of Sciences of the U.S.A. 85: 7177-7181.

Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I. (1987). Expression of a full-length cDNA for the human "*MDR1*" gene confers resistance to colchicine, doxorubicin and vinblastine. Proceedings of the National Academy of Sciences of the U.S.A. 84: 3004-3008.

Vallagussa, P. A. and Bonnadonna, G. (1995). Carcinogenic effects of cancer treatment. In: Oxford Textbook of Oncology: 2348-2358. Ed. M. Peckham, H. Pinedo and U. Veronesi. Published by Oxford University Press.

Van der Zee, A. G. J., De Jong, S., Keith, W. N., Hollema, H., Boonstra, H. and De Vries, E. G. E. (1994). Quantitative and qualitative aspects of topoisomerase I and IIα and β in untreated and platinum/cyclophosphamide treated malignant ovarian tumours. Cancer Research 54: 749-755.

Van der Zee, A. G. J., Hollema, H., De Jong, S., Boonstra, H., Gouw, A., Willemse, P. H. B., Zijlstra, J. G and De Vries, E. G. E. (1991). P-glycoprotein expression and topoisomerase I and II activity in benign tumours of the ovary and in malignant tumours of the ovary, before and after platinum/cyclophosphamide chemotherapy. Cancer Research 51: 5915-5920.

Van Maanen, J. M. S., de Vries, J., Pappie, D., van der Akker, E., Lafleur, M. V. M., Retel, J., van der Greef, J. and Pinedo, H. M. (1987). Cytochrome P-450-mediated *O*-demethylation: a route in the metabolic activation of etoposide (VP-16-213). Cancer Research 47: 4658-4662.

Van Maanen, J. M. S., de Ruiter, C., de Vries, J., Kootstra, P. R., Gobas, F. and Pinedo, H. M. (1985a). The role of metabolic activation by cytochrome P-450 in covalent binding of VP-16-213 to rat liver and HeLa cell microsomal proteins. European Journal of Cancer and Clinical Oncology 21: 1099-1106.

Van Maanen, J. M. S., de Ruiter, C., van der Straat, R., Broersen, J., de Vries, J. and Pinedo, H. M. (1985b). Chemical and biological activation of the dimethoxyphenol ring of etoposide. British Journal of Cancer 52: 463.

Van Maanen, J. M. S., de Ruiter, C., Kootstra, P. R., Lafleur, M. V. M., de Vries, J., Retel, J. and Pinedo, H. M.(1985c). Inactivation of ϕX174 DNA by the ortho-quinone derivative or its reduction product of the antitumour agent VP-16-213. European Journal of Cancer and Clinical Oncology 21: 1215-1218.

Vasey, P. A., Bissett, D., Strolin-Benedetti, M., Poggesi, I., Breda, M., Adams, L., Wilson, P., Pacciarini, M. A., Kaye, S. B. and Cassidy, J. (1995). Phase I clinical and pharmacokinetic study of 3'-deamino-3'-(2-methoxy-4-morpholinyl)doxorubicin (FCE 23762). Cancer Research 55: 2090-2096.

Vaudaux, P., Kiefer, B., Forni, M., Joris, I., Majno, G. and Waldvogel, F. A. (1984). Adriamycin impairs phagocytic function and induces morphologic alterations in human neutrophils. Cancer 54: 400-410.

Verheijen, R., Venrooij, W. V. and Ramaekers, F. (1988). The nuclear matrix. Structure and composition. Journal of Cell Science 90: 11-36.

Versantvoort, C. H. M., Schuurhuis, G. J., Pinedo, H. M., Eekman, C. A., Kuiper, C. M., Lankelma, J. and Broxterman, H. J. (1993). Genestein modulates the decreased drug accumulation in non-P-glycoprotein mediated multidrug resistant tumour cells. British Journal of Cancer 68: 939-946.

Von Hoff, D. D. and Layard, M. W. (1981). Risk factors for the development of daunorubicin cardiotoxicity. Cancer Treatment Reports 65 (suppl. 4): 19-23.

Von Hoff, D. D., Layard, M. W., Basa, P., Davies, H. L. Jr, Von Hoff, A. L., Rozencweig, M., Muggia, F. M. (1979). Risk factors for doxorubicin-induced congestive heart failure. Annals of Internal Medicine 91: 710-717.

Wakelin, L.. P. G., Atwell, G. J., Rewcastle, G. W. and Denny, W. A (1987). Relationships between DNA binding kinetics and biological activity for the 9-aminoacridine-4-carboxamide class of antitumour agents. Journal of Medicinal Chemistry 30: 855-861.

Wall, M. E., Wani, M. C., Cooke, C. E., Palmer, K. H., McPhail, A. T. and Slim, G. A. (1966). The isolation and structure of camptothecin, a novel alkaloidal leukemia and tumour inhibitor from *Camptotheca acuminata*. Journal of the American Chemical Society 88: 3888-3890.

Walsh, S. M., Walley, V. M., Chandra, L., Huan, S. D., Veinot, J. P. and Higginson, L. A. (1995). Potential cardiotoxicity with the use of DuP 491: a case report. Canadian Journal of Cardiology 11: 419-422.

Wang, H. K., Morris-Natschke, S. L. and Lee, K. H. (1997). Recent advances in the discovery and development of topoisomerase inhibitors as antitumour agents. Medicinal Research Reviews 17(4): 367-425.

Wang, J. C. (1996). DNA topoisomerases. Annual Review of Biochemistry 65: 635-692.

Waring, M. J. (1981). Molecular Basis of Drug Action (2nd edition) p258. Ed. Gale, Cundliffe, Reynolds, Richmond and Waring. Published by John Wiley and Sons (London).

Waring, M. (1970). Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs; evidence for molecular models involving intercalation. Journal of Molecular Biology 54: 247-279.

Wassermann, K. (1994). Intragenomic heterogeneity of DNA damage formation and repair: A review of cellular responses to covalent drug-DNA interaction. Critical Reviews in Toxicology 24(4); 281-322.

Wassermann, K., Markovits, J., Jaxel, C., Capranico, G., Kohn, K. W. and Pommier, Y. (1990). Effects of morpholino doxorubicins, doxorubicin and actinomycin D on mammalian DNA topoisomerases I and II. Molecular Pharmacology 38: 38-45.

Watson, J. D., Hopkins, N. H., Roberts, J. W., Argetsinger Steitz, J. and Weiner, A. M. (1987). Molecular Biology of the Gene (4th edition). Ed. J. Reece Gillan. Published by Benjamin/Cummings Inc., California, U. S. A.

Watt, P. M. and Hickson, I. D. (1994). Structure and function of type II topoisomerases. Biochemical Journal 303: 681-695.

Westendorf, J., Aydin, M., Groth, G., Weller, O. and Marquardt, H. (1989). Mechanistic apects of DNA damage by morpholinyl and cyanomorpholinyl anthracyclines. Cancer Research 49: 5262-5266.

Williamson, C. E. and Witten, B. (1967). Reaction mechanism of some aromatic nitrogen mustards. Cancer Research 27: 33-38.

Wilson, W. R., Denny, W. A., Pullen, S. M., Thompson, K. M., Li, A. E., Patterson, L. H. and Lee, H. H. (1996). Tertiary amine N-oxides of bioreductive drugs: DACA N-oxide, nitracrine N-oxide and AQ4N. British Journal of Cancer 74 (suppl. 27): S43-S47.

Wilson, W. R. and Whitmore, G. F. (1981). Cell-cycle stage specificity of 4'-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA) and interaction with ionizing radiation in mammalian cell cultures. Radiation Research 87: 121-136.

Withoff, S., De Jong, S., De Vries, E. G. E. and Mulder, N. H. (1996). Human DNA topoisomerase II: Biochemistry and role in chemotherapy resistance (review). Anticancer Research 16: 1867-1880.

Woessner, R. D., Mattern, M. R., Mirabelli, C. K., Johnson, R. K. and Drake, F. (1991). Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. Cell Growth and Differentiation 2: 209-214.

Wolf, C. R., McPherson, J. S. and Smyth, J. F. (1986). Evidence for the metabolism of mitoxantrone by microsomal glutathione transferases and 3-methylcholanthrene-inducible glucuronosyl transferases. Biochemical Pharmacology 35: 1577-1581.

Wrighton, S. A., Ring, B. J., Watkins, P. B. and VandenBranden, M. (1989). Identification of a polymorphically expressed member of the human cytochrome P-450III family. Molecular Pharmacology 36: 97-105.

Yuan, F., Leunig, M., Huang, S. K., Berk, D. A., Papahadjopoulos, D. and Jain, R. K. (1994). Microvascular permeability and interstitial penetration of sterically stabilized (Stealth)

liposomes in a human tumor xenograft. Cancer Research 54: 3352-3356.

Zamora, J. M., Pearce, H. L. and Beck, W. T. (1988). Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. Molecular Pharmacology 33: 454-462.

Zee-Cheng, R. K.-Y., Podrebarac, E. G., Menon, C. S. and Cheng, C. C. (1979). Structural modification study of bis(substituted aminoalkylamino)anthraquinones. An evaluation of the relationship of the [2-[(2-hydroxyethyl)amino]ethyl]amino side chain with antineoplastic activity. Journal of Medicinal Chemistry 22(5): 501-505.

Zee-Cheng, R. K.-Y. and Cheng, C. C. (1978). Antineoplastic agents. Structure-activity relationship study of bis(substituted aminoalkylamino)anthraquinones. Journal of Medicinal Chemistry 21(3): 291-294.

Zee-Cheng, R. K.-Y. and Cheng, C. C. (1970). Common receptor-complement feature among some antileukemic compounds. Journal of Pharmaceutical Sciences 59: 1630-1654.

Zeller, K. P., Mewes, K., Ehninger, G. and Blanz, J. (1994). Formation of reactive intermediates by cytochrome P-450 mediated oxidation of the anti-cancer drug mitoxantrone. Pure and Applied Chemistry 66(10-11): 2415-2418.

Zhang, K., Mack, P., Wong, K. P. (1998). Glutathione-related mechanisms in cellular resistance to anticancer drugs (review). International Journal of Oncology 12: 871-882.

Zhang, Z. R., Liao, G. T., Nagai, T. and Hou, S. X. (1996). Mitoxantrone polybutyl cyanoacrylate nanoparticles as an antineoplastic targeting drug-delivery system. International Journal of Pharmaceutics 139: 1-8.

Zhang, H., D'Arpa, P. and Liu, L. F. (1990). A model for tumour cell killing by topoisomerase II poisons. Cancer Cells 2(1): 23-27.

Zini, N., Martelli, A. M., Sabatelli, P., Santi, S., Negri, C., Astaldi Ricotti, G. C. B. and Maraldi, N. M. (1992). The 180-kDa isoform of topoisomerase II is localised in the nucleolus and belongs to the structural elements of the nucleolar remnant. Experimental Cell Research 20: 460-466.

Zunino, F. Gambetta, R. and DiMarco, A. (1975). The inhibition *in vitro* of DNA polymerase and RNA polymerases by daunomycin and adriamycin. Biochemical Pharmacology 24: 309-311.

Zwelling, L. A., Kerrigan, D. and Lippman, M. E. (1983). Protein-associated intercalator-induced DNA scission enhanced by estrogen stimulation in human breast cancer cells. Proceedings of the National Academy of Sciences of the U.S.A. 80: 6182-6186.